CELL SURFACE PROPERTIES IN RELATION TO GROWTH AND FORM IN CHINESE HAMSTER OVARY CELLS IN CULTURE

By

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KEY TO ABBREVIATIONS

CHO cells	Chinese Hamster Ovary cells
dB-cAMP	dibutyryl 3':5'-cyclic adenosine monophosphate
Sq 20009	1-Ethyl-4-(isopropylidenehydrazino)-1 H-pyrazolo-[3,4-b] pyridine-5-carboxylic acid, ethyl ester HCl
PB	.01 M sodium phosphate buffer, pH = 7.4
PBS	Phosphate buffered saline
HBSS	Hanks balanced salt solution
SDS	sodium dodecyl sulfate
WGA	Wheat germ agglutinin

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The addition of dibutyryl 3':5'-cyclic AMP (dB-cAMP) to cultures of Chinese Hamster Ovary (CHO) cells induces changes in the appearance and properties of these cells which seem to be the reverse of those that normally occur following transformation of fibroblasts by oncogenic virus. In this study this phenomenon of "reverse transformation" has been examined in detail in four lines cloned from an L-proline-requiring CHO line. The four lines K-1, M-7, 24-2 and H-7, show contrasting, rapid morphological response both to dB-cAMP and to the phosphodiesterase inhibitor Sq 20009. Whereas K-1 and H-7 elongate in presence of dB-cAMP, M-7 does not change in morphology, although it does respond to Sq 20009. 24-2 is normally fibroblast-like in appearance but shows some slight response to both drugs. This system

is therefore an excellent one for comparing cells which show contrasting morphologies and social behavior, yet which are genotypically very similar. An added advantage was that the growth rate and final cell densities of each of the lines were not influenced by the drugs.

The susceptibility of certain of the lines to agglutination by the plant lectins wheat germ agglutinin and concanavalin A was also affected by growth in the presence of Sq 20009 and dB-cAMP although the cells bound similar amounts of lectin under both growth conditions. Each of the clones was agglutinated by both lectins in their normal state. However, whereas K-1, which is morphologically responsive, remained fully agglutinable after treatment with dB-cAMP, H-7 and 24-2 quickly lost their agglutinability. Line M-7 was unaffected by dBcAMP but lost its susceptibility to the lectins following treatment with Sq 20009. In addition, H-7 showed a marked drop in agglutinability in presence of the natural nucleotide 3':5'-cyclic AMP to which it does not respond morphologically. There was not, therefore, an absolute correlation between the morphological responsiveness of the cells and lectin-induced agglutinability. Therefore, these features of the transformed phenotype, morphology and agglutinability, are not necessarily related phenomena.

The rapid losses of agglutinability are prevented by actinomycin D, cordycepin and cyclohexamide, even if dB-cAMP is present. The return to the agglutinable state, however, which also occurs very rapidly is not prevented by these inhibitors. It is concluded that the components responsible for maintaining the non-agglutinable state turnover rapidly.

Neither the plasma membrane protein composition, as determined by electrophoretic analysis on polyacrylamide gels, nor the surface proteins available for lactoperoxidase mediated iodination showed a gross modification of the composition or orientation of surface polypeptides of the CHO cells in the response to dB-cAMP, which could be correlated with any of the other phenomena studied.

In presence of dB-cAMP and Sq 20009, certain of the lines demonstrate an overall reduction in the size of a class of fucose-containing glycopeptides that can be released from their surface with trypsin. This decrease in the size of the glycopeptide is due to a lower content of sialic acid. The change correlated with the losses in agglutinability but not with the morphological responsiveness of the cells. Double-labeling experiments with L-fucose have shown that upon addition of dB-cAMP existing glycopeptides are not modified, but new material reaching the surface is incompletely glycosylated. After removal of the drug, sialic acid can be added to these incomplete glycopeptides. It is proposed that the loss in protein mobility induced by dB-cAMP results in a lowered efficiency in glycosylation at the surface since substrate and enzyme will have less opportunity for contact, and a loss in agglutinability by plant lectins.

Chairman

INTRODUCTION

Chinese hamster ovary cells

Most CHO cells in culture originate from Dr. T. T. Puck's laboratory. They are derived from an original explant and are presumably fibroblast in origin. However, the parental line, CHO-K-1 which was isolated by Kao and Puck (1) does not show a strict fibroblast-like morphology. CHO cells are not believed to be tumorigenic, but they show many of the characteristics of transformed fibroblasts. For example, they have an apparent capacity for unlimited growth, and will grow in soft agar. Furthermore, they show little tendency to orientate in parallel arrays and they do not show contact inhibition of growth (2,3).

The effect of dB-cAMP and Sq 20009 on the intracellular 3':5'-cyclic AMP level

dB-cAMP, an analog of 3':5'-cyclic AMP, recognized as the "second messenger" in the activity of many hormones (4), has been used extensively to induce changes in growth and metabolism of numerous cell lines, including the CHO cells. The latter cells undergo morphological and physiological changes which resemble the reverse of those which occur when cells are transformed by chemical carcinogens or viruses. For example, upon viral transformation, fibroblasts typically lose their elongated shape and assume a more compact morphology (5). They also demonstrate reduced contact inhibition of growth (6,7), become

more agglutinable by plant lectins (8) and show a change in fucose-containing surface glycopeptides (9-12). It is believed that dB-cAMP acts by increasing the effective intracellular concentrations of 3':5'-cyclic AMP. dB-cAMP is reported to be relatively less sensitive to degradation by serum enzymes as compared to 3':5'-cyclic AMP (13-18). It is also an inhibitor of 3':5'-cyclic nucleotide phosphodiesterase (14,19). Further, the less charged dB-analog may be more easily transported across the membrane although this has been disputed (20). It is therefore not surprising that the analog is generally but not universally (21) more effective in inducing "reverse transformation" than the natural nucleotide itself. That uptake is an important factor in determining the effectiveness of 3':5'-cyclic AMP was demonstrated by fusion of lipid-vesicles containing 3':5'-cyclic AMP with cell-membranes (22). The cyclic nucleotide concentration effective in causing a reduction in growth rate was three orders of magnitude smaller when the cyclic nucleotide was introduced into the cell by fusion of lipid-vesicles with the cell-membranes than when it was added to the growth medium. The differences in effectiveness in different cell lines to increase the intracellular 3':5'-cyclic AMP concentrations observed between the cyclic nucleotide and its dibutyryl analog, may well reflect differences in uptake and phosphodiesterase activities between the cell lines.

Some workers have suggested that the effect of dB-cAMP is not due to its acting as an analog of 3':5'-cyclic AMP (21). However, a number of diesterase inhibitors such as theophyline and papaverine and hormones such as prostaglandins and testosterone, which due to their stimulating effect on adenyl cyclase might be expected to increase intracellular

3':5'-cyclic AMP levels, have an effect similar to dB-cAMP (3,23,24). Furthermore, growth stimulation of untransformed cells by protease treatment (25) or by insulin (26,27) decreases intracellular 3':5'-cyclic AMP levels, and these effects can be prevented by dB-cAMP.

Hsie and coworkers have recently published an extensive study on the mode of action of dB-cAMP and its degradation products by CHO-K-1 (20,28). It was shown that dB-cAMP was largely metabolized to two major products: 1) O^2-monobutyryl-cyclic AMP, which in turn was rapidly hydrolyzed, and 2) N^6-monobutyryl-cyclic AMP, which was relatively stable and acted as a competitive inhibitor of one of the 3':5'-cyclic AMP-phosphodiesterases causing intracellular 3':5'-cyclic AMP concentrations to rise. However, even after 4 hours' incubation significant amounts of dB-cAMP were still present. Their work did not rule out the possibility therefore that dB-cAMP was not acting directly as an analog of 3':5'-cyclic AMP. Although its effectiveness may vary from cell line to cell line, it seems unequivocal that dB-cAMP acts by increasing effective intracellular 3':5'-cyclic AMP concentrations.

The effect of intracellular 3':5'-cyclic AMP concentrations on growth rate and pattern

The relationship between 3':5'-cyclic AMP concentrations and cell growth which was alluded to earlier has been investigated in many cell lines. The available evidence strongly suggests that there is a direct relationship between intracellular 3':5'-cyclic AMP levels and cell growth. 3':5'-cyclic AMP levels seem to vary in relation to degree of confluency, growth rate and the physiological state of the cultures. For example, cyclic nucleotide levels rise when the cell reaches confluency, in cells that exhibit contact inhibition of growth, but not

in cells that do not show this phenomenon (29-32). By increasing the intracellular 3':5'-cyclic AMP concentrations through addition of dB-cAMP (33,34) an apparent contact inhibition of growth could be induced in transformed fibroblasts. Some investigators suggested that the rise in 3':5'-cyclic AMP concentrations noted at confluency only reflected a depletion of serum factors and is not correlated with contact inhibition of growth (35). However, upon addition of trypsin (25,31), insulin (25) or serum stimulation (35), 3':5'-cyclic AMP levels first fall before cell division occurs. When 3':5'-cyclic AMP levels were measured in cells at different stages of the cell cycle it was found that the cAMP levels were lowest during mitosis and rose again during the G-1 phase (25,36). 3':5'-cyclic AMP has also been implicated in the regulation of mobility of fibroblasts. When these cells were grown in the presence of dB-cAMP or prostaglandin E, which also increases intracellular 3':5'-cyclic AMP (37,38) levels the cells became less mobile (39). Furthermore, the adhesion of these cells to the substratum was increased (40) when grown in the presence of dB-cAMP or theophyline. The ordered growth patterns observed in CHO cells upon addition of dB-cAMP to the growth medium (2) may also be due to increased cell-cell recognition and to reduced mobility and stronger adhesion to the substratum of dB-cAMP treated cells. The opposite effects were seen in fibroblasts grown in the presence of insulin. These lost their ordered growth pattern and were freed from contact inhibition of growth while their 3':5'-cyclic AMP levels fell (30).

Another aspect of cell growth in the presence or absence of drugs increasing 3':5'-cyclic AMP concentrations, is the change in transport observed for many nutrients (41-44). Especially important in relation

to this study is the fact that different cell lines show a contrasting effect of these drugs on thymidine uptake. The uptake of thymidine was reported to increase in SV40-CV-1 (43), a monkey kidney cell transformed by SV40 while it decreased in CHO cells (44) upon addition of cyclic AMP to the medium. From this observation it is clear that incorporation of ^{3}H-thymidine in DNA is not a good measure of the growth rate of cells treated with different drugs.

When CHO cells are grown in the presence of dB-cAMP or other drugs or hormones which increase intracellular 3':5'-cyclic AMP levels, they undergo a process which was termed "reverse transformation" by Hsie and Puck (2). They typically revert back from a compact morphology to a more elongated form which resembles "normal" fibroblasts. They align in parallel arrays, they show a decreased agglutinability by plant lectins and an increase in synthesis of collagen (3) and of acid mucopolysaccharides (45). There is also a shift in fucose-containing glycopeptides at the cell surface towards components of lower molecular weight as estimated by chromatography on Sephadex G-50 (46,47). CHO cells can be enucleated by treating them with cytochalasin B, a drug which disrupts microfilaments in the cells (48-50). These enucleated CHO cells contain all cell-organelles, regain their normal shape and display some motility after enucleation. They also elongate upon treatment with dB-cAMP (50). These observations suggest that cellular elongation in response to dB-cAMP is independent of nuclear events (i.e. transcription of RNA). This is also consistent with experiments indicating that elongation induced by dB-cAMP is independent of protein or RNA synthesis (51).

The role of 3':5'-cyclic AMP in cellular growth and morphology prompted investigators to study the possible role of an altered 3':5'-

cyclic AMP metabolism in tumor cells which have abnormal growth patterns. Lower levels of cyclic AMP were indeed found in several transformed lines as compared to their normal counterparts (52,53) and it was suggested that this might be the basis of their altered growth properties. The most convincing results were obtained with several cell lines transformed by a temperature-sensitive mutant of the Rous Sarcoma Virus. When these cells are grown at the permissive temperature, i.e. when the viral function is expressed, but not at the nonpermissive temperature, there is a decrease in 3':5'-cyclic AMP content because of a decrease in adenyl cyclase activity (54,55). In another study using a temperature sensitive mutant of SV40, the growth of the transformed cell was temperature sensitive, however, although the 3':5'-cyclic AMP levels were lower in these cells than in the parental line, they were not temperature sensitive (56). dB-cAMP also prevents the induction of DNA synthesis induced by adenovirus type 12 in BHK cells arrested in G-1 (57) and reduces the tumorigenicity of Celovirus transformed CHO cells (24). Adenyl cyclase, the enzyme which catalyzes the synthesis of 3':5'-cyclic AMP from ATP, is part of the plasma membrane (58). Its activity can be modified by extracellular agents which bind to plasma membrane receptors (58-60). Insulin, which affects adenyl cyclase, can be immobilized on Sepharose beads and still retain its effectiveness even though it cannot enter the cell. This indicates that the interaction of insulin with superficial membrane structures alone may suffice to affect adenyl cyclase activity (59).

"The fluid mosaic model" of the plasma membrane

A number of models have been suggested to explain the structures and properties of the plasma membrane (see reviews 61-67). The one most

accepted has been proposed by Singer and Nicolson (68). They recognized that the membrane was not a rigid structure, but a dynamic, fluid organelle. Figure 1 shows the model they proposed with modifications and additions accepted since its publication. Basically the membrane is believed to consist of a lipid bilayer in which the lipid molecules are arranged with their hydrophobic aliphatic chains pointing inwards towards each other and the polar heads forming the outer edges. This structure resembles a flattened micelle. Globular proteins are distributed along and within this bilayer. Some only penetrate partially into the bilayer, others span the entire membrane (69,70).

Although it was elegantly shown that membranes are fluid (71), other experiments provide evidence that membrane proteins are not completely free to move and can also assume a non-random distribution that is characteristic for a particular cell type or physiological state and which can be modified (72-75). For example, different topographic changes can be induced in transformed cells as compared to the normal parental cell lines using plant lectins such as concanavalin A (76-79). For while the Con A binding sites on transformed cells formed clusters, there was a more random distribution on the normal cell types. Where cells were in contact (i.e. agglutination sites) clustering was particularly evident. It was proposed that the lectin molecules were involved in forming multiple cross-links in these regions. It seems, therefore, that the lectin receptors on normal cells migrate less readily in the plane of the membrane than in their transformed counterparts. This implies the existence of forces that limit free diffusion of membrane proteins and suggests that there may be differences in the nature or effectiveness of restraining or limiting forces on membrane proteins.

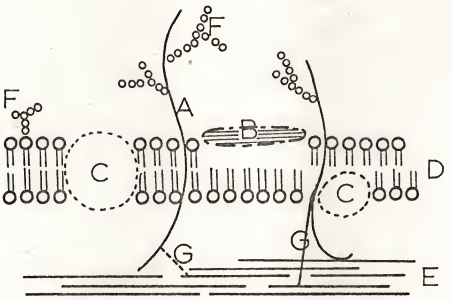


Figure 1. A schematic representation of the Fluid Mosaic Model of plasma membranes.

A - Proteins carrying carbohydrate moieties.

B - Trypsin labile protein, may be connected in some way to A.

C - Membrane particles.

D - Lipid bilayer.

E - Microtubular framework.

F - Carbohydrate moieties.

G - Microtubules or microfilaments connecting A with E.

There were not sufficient differences in lipid composition to produce overall differences in membrane fluidity.

A molecule with a polar region probably cannot move from the inside to the outside of the bilayer or vice versa (80). This would be thermodynamically unfavorable since the polar region would have to pass through the non-polar bilayer. However, as seen before, the lipid bilayer allows the globular proteins to diffuse laterally in the plane of membrane, subject to the restraints mentioned above.

Some membrane proteins extend outward from the plane of the membrane. These proteins often carry carbohydrate branches on a protein backbone, while lipids are completely embedded within the bilayer, with only the polar head, which carries the carbohydrate moieties extending outward. The different saccharide residues are localized mainly on the outer face of the membrane (81,82). These cell surface molecules are believed to carry some of the antigenic determinants and many hormone receptors of the cell (64). There is some evidence that these glycoproteins are linked on the inner face of the plasma membrane to parts of an internal framework of microtubules and microfilaments, as indicated in Figure 1.

Microtubules and microfilaments are structural components visible only by electron microscopy. Both are present in cultured fibroblasts (83-87). Microtubules are composed of polypeptide subunits called tubulin. Colchicine and vinblastin disrupt these microtubules. dB-cAMP on the other hand appears to induce the polymerization of tubulin into microtubules without increasing the total amount of tubulin present in the cell (52). The exact mechanism by which dB-cAMP induces polymerization is not known, although it may involve phosphorylation of the protein

dimer (88). Both actin and myosin were found in the microfilament bundles, and it is believed that they can provide the motility and contractility of the cell (87). In fibroblasts microtubules are mainly aligned in parallel to the long axis of the cell. When cells are treated with compounds which disrupt microtubular structure, the cells lose their elongated shape, suggesting that microtubules are involved in the maintenance of cell morphology.

Linkages between microtubules and membrane-bounded vesicles have been detected by electron microscopy (89). This may explain the decrease in collagen secretion and a reported increased number of deleted Golgi vesicles, agents providing the directive channels for movement of secretory vesicles to the cell surface upon disruption of the microtubules (90). Microtubules have been implicated as the restraining forces involved in agglutinin receptor-protein mobility (91-97), and in phagocytosis (97). Upon transformation a decreased amount of membrane associated actin in fibroblasts have been reported, although the total amount of actin in the cell remained constant (98). The myosin content of transformed rat kidney cells was decreased to half that of normal cells. However, dB-cAMP did not affect the myosin content, although it did affect the morphology of the cell (85). It seems that the microtubules and microfilaments form a filamentous subcellular microarchitecture which may be very important in several cell functions. However, the exact mode of involvement is not yet known.

Complex saccharides at the cell surface

Under electron microscope many cells have a fuzzy region around their periphery. Histochemical observations suggested that was largely carbohydrate in nature (99,100,70,71). Evidence suggests that much of

the carbohydrate is located at the cell surface and protrudes out from the membrane (71,101). However, in the literature there is no adequate distinction between the true cell membrane proteins discussed above and the extracellular coat. The latter is probably loosely attached, secreted material rich in mucopolysaccharides such as hyaluronic acid, chondroitin sulfate, and heparan sulfates (100). A number of changes in the cell surface carbohydrates have been reported to accompany transformation, the most well defined are discussed below.

a) Mucopolysaccharides. Using staining techniques it was observed that the surface coat of transformed cells was thicker than that of normal cells (102-104). On the other hand, a decreased rate of synthesis of acid mucopolysaccharides was observed, which could be counteracted by addition of dB-cAMP and theophylline to the growth medium.

Others have reported a tenfold increase in [^{3}H]-glucosamine incorporation into the hyaluronic acid fraction following viral transformation but no increased incorporation in the sulfated fraction of the cell coat (105). The same authors reported an increase in the average molecular size of cell coat molecules. By contrast, other authors using different cell lines have reported contradictory results (106). It seems that there is no consistent pattern of altered mucopolysaccharides when cells become malignant.

b) Glycolipids. Changes observed in this class of molecules include a general trend toward simplification, e.g. size reduction of carbohydrate moieties upon transformation (107-110) and quantitative changes in normal cells but not transformed cells during the cell cycle (111). However, some investigators report no changes upon transformation (112-114).

Recently it was shown that upon labeling the cells by cell surface specific methods, transformed cells lacked label in "galactoprotein" present in normal cells but did have label in a glycolipid which was not labeled in normal cells (115-116). It was also shown that when cells were grown in the presence of dB-cAMP or dextran sulfate, the glycolipid labeling pattern of a transformed cell became indistinguishable from that of a normal cell (117).

Results of comparative experiments on glycolipid content of normal and transformed cells are difficult to compare because of the effect of growth condition and cell density on glycolipid content (118). Furthermore, an analysis of glycolipid content in a number of contact inhibited clones showed that there were no consistent changes in lipid patterns related to degree of malignancy (119). Again results have been confusing and it is not justifiable at the present time to make any absolute correlations between surface glycolipid composition and the transformed state.

c) Glycoproteins. Several differences were observed in membrane glycoproteins after transformation. These include an increase in apparent molecular weight of the carbohydrate moieties of certain surface glycoproteins, those that contained L-fucose (9-12), the disappearance of a high molecular weight glycoprotein (120,121), and altered glycosyl transferase activities at the cell surface (122-125).

Transformed cells have certain glycoproteins of average higher molecular weight at their cell surface than do normal cells. It was shown that this difference is due to an increased amount of sialic acid on the fucose-containing glycoproteins (10). Warren and his associates also reported that transformed cells have a fivefold higher sialic acid

transferase activity than do normal cells. This enzyme was specific for particular desialated glycopeptides and was not active towards desialyzed fetuin, the normal substrate for such assays.

Other workers, in contrast, have shown that the overall glycosyl transferase activities of normal cells were increased as compared to transformed cells (122,123). Furthermore, normal cells could only transfer galactose to adjacent cells, whereas transformed cells (126) and mitotic normal cells (127) can transfer galactose to receptors on the same cell, i.e. endogenous receptors. It has been suggested that these surface enzymes play a role in contact inhibition of growth. Complexes between a glycosyl transferase on one cell and an incompleted carbohydrate chain, the substrate, on adjacent cells are visualized as forming the corss-links. This has been disputed by other investigators (128). Finally it has been reported that transformed cells lacked a high molecular weight glycoprotein when plasma membrane polypeptide compositions of these and normal cells were compared by polyacrylamide gel electrophoresis (120,121). This protein had a molecular weight greater than 200,000 and was removed from normal cells by brief treatment with trypsin. In cells transformed by temperature sensitive virus it appeared only at the permissive temperature. A similar protein could be induced in neuroblastoma cells grown in the presence of dB-cAMP (129). Under these conditions the neuroblastoma cells underwent morphological differentiation, i.e. growth of long processes resembling neurites was induced. At present the functional significance of such a protein is unclear.

d) The architecture of the cell surface. The orientation and distribution of the cell surface glycoproteins in the plasma membrane

may be very important in determining cell surface characteristics. Useful methods of studying these parameters are: 1) the agglutination of cells by plant lectins. Glycoproteins characteristically bind to plant lectins such as concanavalin A (130,131). Although it was determined that transformed cells do not bind significantly more lectins (132,133), transformed cells are more agglutinable than are their normal counterparts. As was pointed out earlier, investigators believe that this is due to a higher mobility of the binding sites, and hence glycoproteins in the membranes of transformed cells; 2) chemical labeling techniques. In these methods the radiolabel was supplied either in an appropriate precursor, through membrane impermeable reagents which reacted with available groups on surface molecules (134,135) or by using enzymes which presumably do not enter the cell to oxidize surface molecules, and subsequently reducing these same molecules with [^{3}H]-sodium borohydride (136,137). An advantage of supplying the label through precursors is that one can use contrastingly labeled compounds. The advantages, disadvantages and molecular bases for the methods employed in this study are detailed in the relevant section of the Materials and Methods chapter.

Concluding statement

It is clear from the preceding discussion that although differences have been obtained between normal cells and their transformed counterparts, results have often been contradictory. In part this may be due to "genetic drift," since transformed cells in culture are genetically unstable. Therefore, many invalid comparisons have probably been made between cells which are genetically quite dissimilar. The best systems devised up to now for studying changes accompanying transformation have

been cells transformed by temperature sensitive oncogenic viruses. In this case differences in cell surface composition, agglutinability and morphology have consistently been observed between cells grown at the permissive and non-permissive temperatures. However, even in these experiments it requires at least a full cell cycle, frequently 24-30 h, before the surface becomes "modulated" from the transformed to the normal phenotype.

In the experiments described in this dissertation I have chosen to work with a series of Chinese Hamster Ovary lines, some of which respond morphologically to the dibutyryl derivative of 3':5'-cyclic AMP. The main aim of these experiments was to study the changes in cell surface which accompany these morphological changes. In particular, these lines seemed to be an excellent model for comparing groups of cells which were genetically similar, but whose growth and pattern of growth had been dramatically modified. In addition it seemed that they might provide a system for studying the surface events that normally accompany transformation, since the treated cells have been reported to: a) lose their agglutinability by plant lectins, b) show changes in cell surface glycopeptide composition reminiscent of the reverse of those that normally occur following transformation. In addition, there were the following advantages to this system: a) some of the lines used do not respond morphologically to dB-cAMP, so that the biochemical events responsible for the change in morphology might be distinguished from the cell surface events using the different cell variants, b) as we shall show later, some of the changes occur both rapidly and synchronously, c) the growth rate and final cell densities of the CHO cells are unaffected by the presence of dB-cAMP, so that comparisons can be made between cells in a similar state of growth.

MATERIALS AND METHODS

Materials

Chemicals and radiochemicals

The chemicals and radiochemicals used in this study are listed with their source in Table 1 and Table 2 respectively. Table 2 also lists the specific radioactivity and labeled position of the radiochemicals.

Purification of plant lectins

Wheat germ agglutinin was prepared by a modification of the method of Nagata and Burger (138) from commercially available wheat germ.

Unprocessed whole wheat germ was ground fine in a mill. The rest of this procedure assumed a starting quantity of about 100 grams of wheat germ.

The powder was extracted with a liter of 0.05N hydrochloric acid for 1 hour at room temperature. The residue was centrifuged at 15,000 x G for 20 minutes.

The extract, brought to 35% ammonium sulfate saturation by slow addition of the solid salt, was kept stirring for 1 hour in an ice slurry (4°C) and centrifuged for 20 min at 15,000 x G. The precipitate was resuspended in 200 ml of 0.05N hydrochloric acid.

Insoluble material was removed by centrifugation. n-Butanol was added dropwise to give a final concentration of 20% (v/v) with constant stirring at room temperature. After one hour of stirring, the mixture

TABLE I. Sources of chemicals

Compound	Manufacturer
N,N,N',N',-tetramethylethylene diamine	Eastman Kodak Company Rochester, New York
Ammonium persulfate	ibid
Cyanogum 41	ibid
1-ethyl-4-(isopropylidene-hydrazino)-1H-pyrazola-[3,4-b] pyridine-5-carboxylic acid, ethyl ester	Squib Institute for Medical Research Princeton, New Jersey
Cordycepin	Sigma Chemical Co. St. Louis, Missouri
Cyclohexamide	
Actinomycin D	
N^6,O^2-dibutyryl adenosine	
3':5'-cyclic monophosphoric acid	
Galactose oxidase	
Fluoresceine mercuric acetate	
Streptomyces protease	
Trypsin	
Chymotrypsin	
Ovalbumin	
Bovine serum albumin	
Neuraminidase	
Lactoperoxidase	Calbiochem International La Jolla, California
Sephadex (G-50)	Pharmacia Fine Chemicals Piscataway, New Jersey
Diethyl amino ethyl cellulose	
Concanavalin-A-sepharose	Bio-Rad Laboratories Richmond, California
Biorad P-2	
Glutamine	Grand Island Biochemical Co. New York
Mc Coy's 5A medium	
Fetal calf serum	
Antibiotic-antimycotic	
Trypsin	

TABLE 2. Sources and specific activities of radiochemicals

Compound	Label	Specific Activity	Manufacturer
L-fucose	[1-^{14}C]	57 Ci/mol	Amersham-Searle Arlington Heights, Ill.
	[1-^{3}H]	1 Ci/mmol	
L-leucine	[4,5-^{3}H]	38 Ci/mmol	
	[U-^{14}C]	348 Ci/mol	
Sodiumboro-hydride	[^{3}H]	570 Ci/mol	
Acetic anhydride	[1-^{14}C]	24 Ci/mol	
Acetic anhydride	[^{3}H]	51 Ci/mol	
Thymidine	Methyl[^{3}H]	48.5 Ci/mmol	New England Nuclear Boston, Massachusetts
Sodium iodide	[^{125}I]	17 Ci/mg	International Chemical and Nuclear Waltown, Massachusetts

was centrifuged at 5,000 x G for 30 min. The uppermost n-butanol phase and fluffy interphase were removed by suction followed by filtration. Lower clear water-phase was dialyzed against 2 liters of 0.05N hydrochloric acid overnight.

Ammonium sulfate was added to 35% saturation as in step 3, and centrifuged as above. The precipitate was resuspended in 20 ml of 0.05N hydrochloric acid and dialyzed extensively against 0.01M Tris-HCl buffer, pH 8.5 overnight.

The dialysate was centrifuged to remove insoluble material and applied to a DEAE-cellulose column (2.5 x 20 cm) which was equilibrated with 0.01M Tris-HCl buffer, pH 8.5. Elution was performed with approximately 300 ml of the buffer, with a flow rate of 0.5 ml/min. Wheat germ agglutinin was not adsorbed on the cellulose. Active fractions were pooled, dialyzed against distilled water and lyophilized.

Concanavalin A was prepared from jack bean meal (Sigma Chemical Company, St. Louis, Mo.) by a modified procedure of Agrawal and Goldstein (139). In this procedure 500 g of jack bean were stored overnight at -90°C in order to make the seeds brittle.

200 g aliquots of the frozen bean were then ground (at 4°C) to a fine white powder in a mechanical tissue grinder. The powder was passed through a series of fine mesh screens to remove any large pieces of the seed coat. The powder was extracted for 12 hours in 0.15M NaCl (4:1 (w/w), NaCl:powder) at 4°C. After 12 hours the residue was spun down at 3400G and the supernatant collected. The remaining residue was re-extracted with 0.15M NaCl at 4°C for 12 hours and the supernatant decanted after centrifugation.

The extract was then brought to 40% saturation with solid ammonium sulfate. The precipitate was spun down at 10,000G and discarded. The supernatant was then brought to 60% saturated ammonium sulfate, the precipitate collected and resuspended in 1.0M NaCl. The precipitate was then dialyzed against distilled water for 12 hours at 4°C. The dialysate was added to 1 liter of Sephadex G-75 in PBS, and stirred for 1 hour at 4°C. This solution was poured into a column, which had a void volumn of 120 ml. The column was eluted with 0.1M phosphate buffered saline (pH 7.2). 2.0 ml fractions were collected with an Isco fraction collector and monitored for protein at 280 mμ in a Gilford Spectrophotometer. Most of the protein was removed at the void volume. After the column had been washed free of extraneous protein, the elutant was changed to 0.1M glucose in 0.1M phosphate buffered saline (pH 7.2). Con A, which had been bound to the dextran, came off as a single, symmetrical peak. The fractions containing the concanavalin A were collected, dialyzed overnight against 0.1M phosphate buffered saline (pH 7.2) and stored desicated below 0°C.

500 g of jack bean gave a final yield of approximately 3.0 g of concanavalin A.

Cells

Chinese Hamster Ovary (CHO) cell lines K-1, K-1-M-7 and K-1-24-2 were obtained from Dr. Abraham Hsie, Oak Ridge National Laboratory. The other line (clone H-7) originated in the laboratory of Dr. R. M. Humphrey, Texas Medical Center, Houston. All these cells were derived from the original L-proline requiring CHO clone K-1 of Kao and Puck (1).

Maintenance of Cell Lines

The cells were grown in 75 cm^2 Falcon flasks, Bioquest, Cockeysville MD, in 20 ml McCoys 5A medium, supplemented by 10% v/v fetal calf serum, 2 mM Glutamine and an antibiotic antibicotic mixture with a final concentration of 10^4/liter penicillin, 83.3 meg/l Fungionone and 10^3 meg/l streptomycin. The cultures were routinely passaged 1:10 upon reaching approximately 90% confluency. Trypsin solution, (0.25% w/v Hank's balanced salt solution) was used to free the cells from the plastic. For growth, the temperature was maintained at 37°C in a moist incubator in which the CO_2 tension was 5%.

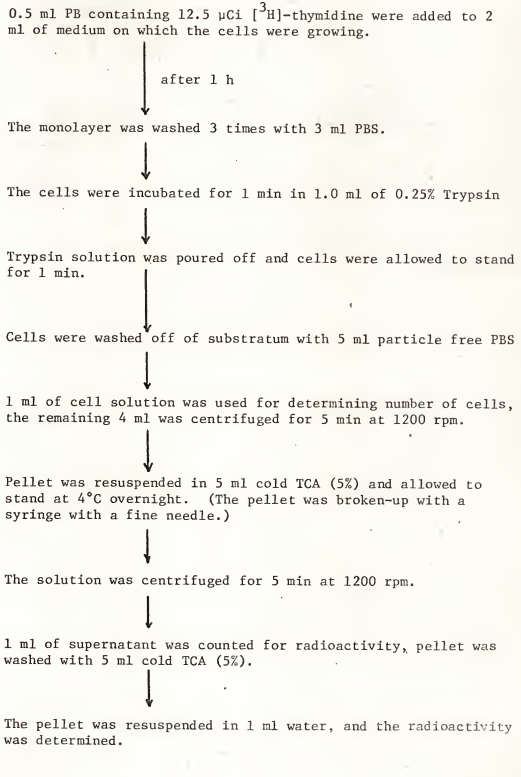
Histological and Microscopic Methods

Cells were grown in Falcon dishes. The drugs were added at the specified concentrations, under sterile conditions. When the cells reached 60-80% confluency, the medium was discarded and the plates were washed free of medium with PB. The cells were fixed for 15 min in 10% (v/v) formaldehyde-PB, and were dehydrated by sequentially incubating them for 5 min in 20, 40 and 60% ethanol-water. The cells were then stained for 15 min with 0.1% (w/v) Toluidine Blue in 70% ethanol-water. The excess stain was washed off with PB and the monolayers were allowed to air dry overnight. A drop of immersion oil was placed on the cells and covered with a coverslip. The cells were photographed under a Wild Heerburg microscope, with camara attachment. A Mamayia/Sekoz TL-500 camara and Kodak Tri-X 135 Pan film were used.

Measurement of the Incorporation and Uptake of [^{3}H]-Thymidine

Cells were grown in the presence or absence of 1 mM dB-cAMP in T-20 Falcon flasks. The procedure used to determine incorporation of

Fig. 2. Flow chart demonstrating the procedure followed in measuring thymidine uptake and incorporation in CHO-cells.



[^{3}H]-thymidine is detailed in Figure 2. When the culture reached the desired density, a certain time after plating, [^{3}H]-thymidine was added for 1 hour. The radioactivity was washed off and the cells were removed from the flask. An aliquot was used for counting cell number, the remainder of the cells were broken and the DNA precipitated with cold trichloroacetic acid, and radioactivity in DNA was determined. An aliquot of the supernatant was counted as a measure of soluble thymidine and uptake of [^{3}H]-thymidine.

Radiochemical Labeling Technique with Precursors

In order to compare the proteins or glycoproteins of the plasma membrane of the cell lines, one line was grown on an appropriate [^{3}H]-labeled precursor, while the other line was grown on the contrasting [^{14}C]-precursor. For studying protein composition radiolabeled L-leucine was employed. For glycoproteins either L-fucose or D-glucosamine were the chosen precursors. When the cultures reached approximately 80% confluency, either plasma membranes were isolated, or the cells were trypsinized for the study of the surface components released by the enzyme. Contrastingly labeled samples from the different lines, or from the same cell line grown under different experimental conditions (e.g. $\pm$ dB-c-AMP) were then mixed and processed together.

Trypsinization of cells

Cells were grown in roller bottles containing 50 ml McCoy's 5A medium, and on medium containing either dB-c-AMP, Sq 20009 or other drugs at the concentrations indicated, in the presence of either [^{3}H]- or [^{14}C]-fucose. These bottles were rotated at 2 rpm and maintained at 37°C. Upon reaching 80% confluency, the cells were rinsed

with 20 ml of PBS, and rotated at 2 rpm for 8 min at 37°C in 10 ml of 0.25% (w/v) Trypsin-PBS. The cells were centrifuged for 3 min at 800 rpm and the supernatants of the two samples with contrasting labels were mixed. The mixed solution was heated at 100°C for 2 min to inactivate the trypsin and digested with pronase to hydrolyze the remaining peptide material. The digest was dried, redissolved in 4 ml water and filtered through glass wool to remove large particles of insoluble precipitate.

Column-Chromatography of Trypsinates

Molecular sieve chromatography

The filtered trypsinate was desalted on a column of Bio-gel P-2. The eluant was water, and the elution rate 50 ml h^{-1}. In each case 55-60% of the total radioactivity appeared as a peak coinciding with the void volume of the column. The remaining radioactivity was largely free in the form of L-fucose and eluted in the included volume of the column. The material collected at the void volume was concentrated to 2 ml and chromatographed on a column of Sephadex G-50 (90 x 1.5 cm; void volume 55 ml). The elution rate was 0.52 ml min^{-1} and each fraction had a volume of 2.6 ml. The column was equilibrated with PB and this buffer also served as eluant.

Ion-exchange chromatography

The fractions 20 to 50 obtained through G-50 molecular sieve chromatography were concentrated and desalted on the Bio-gel P-2 column described in the previous section. The fraction eluting in the excluded volume was collected and concentrated to 4 ml. This sample was applied to a diethylamino-ethyl-sephadex column (7 x 0.9 cm) in water. The column was washed with 50 ml of water after which a salt

gradient was applied. This gradient ranged from 0 to 0.5M, sodium chloride in 0.01M Tris-HCl at pH 8.2 and was non-linear. The gradient was made by connecting 3 chambers in series. The first two chambers contained only 100 ml of buffer, while the last chamber contained 100 ml 0.5M sodium chloride in the buffer. Five ml fractions were collected and 2 ml of each fraction were used to determine radioactivity.

Hydrolytic Procedures Used in Analysis of Carbohydrates

Removal of N-acetyl neuraminic acid using neuraminidase

Hydrolysis of whole cells. A confluent roller bottle containing cells labeled with [^{3}H]-fucose was washed with PB and incubated for 45 min at 37°C with 20 ml PB containing 2 units of neuraminidase. The cells were then trypsinized. This trypsinate was mixed with the trypsinate of a control culture labeled with [^{14}C]-fucose and co-chromatographed as described.

Hydrolysis of carbohydrate moieties. The fractions 20 to 50 obtained from G-50 molecular sieve chromatography were dried under vacuum, desalted on a Bio-gel P-2 column as before, and evaporated to dryness again. The sample was then dissolved in 3 ml of 0.01M, sodium acetate buffer, pH 5.4. 0.2 units of neuraminidase were added and the mixture was incubated for 20 hours at 37°C. This sample was re-chromatographed on the G-50 column, or fractionated by ion-exchange chromatography.

Removal of N-acetylneuraminic acid using hydrochloric acid

The fractions 20 to 50 obtained from G-50 molecular sieve chromatography were dried under vacuum, desalted on a Bio-gel P-2 column as before, and dried again. The sample was then incubated in 1 ml 0.1N hydrochloric acid at 80°C for 1 hour. 3 ml of PB was added at this

time. This final mixture was re-chromatographed by gel filtration on the G-50 column, or by ion-exchange.

Analysis of label

The fractions 20 to 50 obtained from G-50 molecular sieve chromatography were dried under vacuum, desalted on a Bio-gel P-2 column and dried as before. The sample was then incubated in 5 ml 0.1N or 0.5N hydrochloric acid at 100°C for 24 or 48 h. The hydrolyzate was evaporated to dryness and dissolved in 200 µl water. This sample and several reference standards were spotted 1 inch apart on a sheet of Whatman number 1 chromatography paper. Descending chromatography was performed using a solvent system of butanol, acetic acid and water in a ratio of 37:25:9, by volume. A strip containing the sample was cut from the chromatogram. The rest was stained to determine the relative mobility of the standards. The strip containing the sample was scanned using a Packard Model 7201 Radiochromatogram Scanner, then cut in 1 cm strips perpendicular to the direction of separation, and the radioactivity in each was determined by liquid scintillation counting.

Gel Electrophoretic Techniques

Electrophoresis

Polyacrylamide gels were prepared according to the method of Laemmli (140), using the buffer systems of Davis (141). The polyacrylamide gel consisted of two sections: (1) a large-pore gel, 5% (w/v) polyacrylamide with 5% (w/w) bis-acrylamide-polyacrylamide in which electrophoretic concentration takes place (stacking-gel), and (2) a small-pore gel in which electrophoretic separation takes place (running gel). The gels varied in polyacrylamide concentration but always contained 5% (w/w) bis-acrylamide-polyacrylamide. A purified mixture of polyacrylamide

and bis-acrylamide at this ratio (Cyanagum) was purchased from Eastman Kodak, Inc. The diameter of the gels was always 5 mm i.d. Therefore, the length of the gel varied with the volume of gel solution in the glass tubes. For the large-pore gel, 0.3 ml gel solution was used. The gels were polymerized chemically using tetramethylene-diamine and ammonium persulfate. The buffers used are given in Table 3.

The glass tubes were tightly closed at one end with flat topped rubber stoppers. These tubes were arranged vertically and filled with the appropriate solution volume of running gel. A layer of water was carefully layered on top of this solution and the gel was allowed to polymerize. The water was then removed, and 0.3 ml of stacking-gel (large pore gel) solution was added, carefully overlaid with water, and allowed to polymerize.

A Buchler Analytical Temperature Regulation Polyacrylamide Gel Electrophoresis Apparatus was used for the electrophoretic separation (see Figure 3). The same buffer, compartment buffer in Table 3, was used in both upper and lower chambers. Solid crystals of sucrose were added to the sample solution in order to increase its density and 100 µl to 200 µl of the sample, containing approximately 100 µg of protein was layered on the upper surface of the stacking gel. Electrophoresis was carried out by applying a current of 3 mA per tube, until the sample had entered the stacking gel. The current was then increased to 6 mA per tube. One gel, a control, was loaded only with the dye marker bromphenol blue. When the dye reached a point approximately 0.5 cm from the bottom of tube, the power was switched off. This took approximately 60 min for a 6.5 cm gel and 90 min for a 10.0 cm gel.

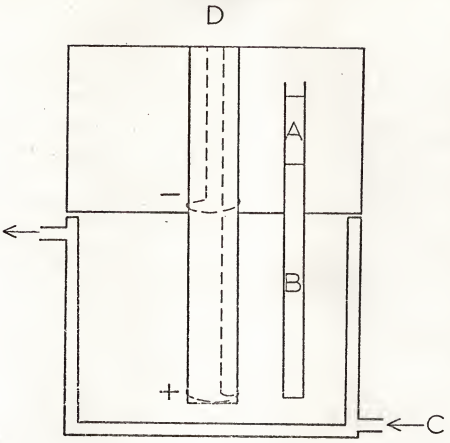


Figure 3. A diagram of the Buchler Analytical Temperature Regulated Polyacrylamide Gel Electrophoresis Apparatus.

A - Stacking gel, B - Running gel

C - Inlet for temperature regulating water

D - Electrical connections

TABLE 3. Buffers used in polyacrylamide gel electrophoresis

Compartment Buffer pH 8.3	.38M Glycine .049M Tris	.1% (w/v) SDS .1% (v/v) β-mercaptoethanol
Stacking Gel Buffer pH 7.6	.00612M Tris .0032M Phosphate	.1% (w/v) SDS
Running Gel Buffer pH 8.9	.375M Tris .006M Chloride	.1% (w/v) SDS

The gels were immediately removed from the glass tube by gently rimming them with a fine needle through which a thin stream of water was passed.

Staining and destaining procedures

Prior to staining the gel, the SDS was removed by shaking the gel in ethanol-acetic acid-water (40:10:50 v/v) overnight. This procedure also pre-"fixed" the protein, thus eliminating diffusion. The gels were stained for protein using 0.125% (w/v) Coomassie blue in the same ethanol-acetic acid-water mixture for 1 hour and diffusion destained in ethanol-acetic acid-water (10:7:83 v/v) until all excess stain was removed.

Gel slicing

Two methods were used for fractionation of the gels. Each gave comparable results.

(1) The gels were fractionated using an "Autogeldivider" (Savant Instruments, Hicksville, NY), into approximately 60-80 fractions per gel. The crushed gel fractions were left overnight in 0.5 ml of water and their radioactivity content determined after addition of 5 ml of toluene-Triton X-100 scintillant (142).

(2) The gels were stained with Coomassie blue. They could then be photographed if desired. They were then frozen in a Revco ultra low temperature freezer at -70°C, before being sliced into 1 mm pieces, using a guillotine of razor blades. Each slice was incubated overnight in 0.5 ml of 30% hydrogen peroxide-water at 60°C in glass scintillation vials in order to solubilize the gel. Their radioactive content was determined after addition of 5 ml of toluene Triton X-100 scintillant.

External Labeling of Cells

These methods are based on a reaction of non-penetrating reagents with reactive groups exposed at the outer surface of the cell. In all of the experiments the cells were grown until approximately 90% confluent, i.e. they were still in log phase growth, before treatment.

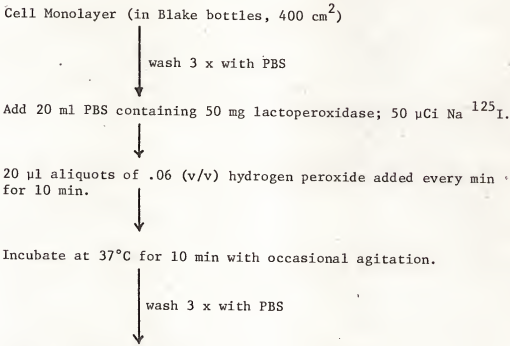
Iodination of the lactoperoxidase technique

This is based on a modification of the method first described by Phillips and Morrison (137), and is relatively specific for tyrosine residues. The detailed mechanism of the reaction is still not understood, but leads mainly to the formation of monoiodo-tyrosine.

Since lactoperoxidase is a relatively large molecule, it does not easily penetrate the membrane and ^{125}I is believed to be incorporated into proteins projecting out of the lipid bilayer. The procedure is outlined in the flow chart shown in Figure 4. After thoroughly washing the monolayer, the cells were covered with buffered saline containing the enzyme and 50 µCi Na ^{125}I. Hydrogen peroxide was then added to initiate the reaction. After incubation, the cells were washed and plasma membranes isolated by the method of Barland and Schroeder (143). In later experiments I attempted to reduce the amount of non-specific ^{125}I introduction into lipid by first treating the cells with sodium borohydride. I_2 and NaI can react with the double bonds of unsaturated fatty acids (144). I reasoned that reduction of these bonds might render the labeling procedure more specific for proteins.

It should be emphasized that the above reactions seem sufficiently mild that labeled cells continue to grow with no measurable reduction in generation time after treatment.

Fig. 4: Flow chart demonstrating the procedure for lactoperoxidase mediated iodination of cell surfaces.



Labeling by the galactose oxidase technique

This method is based on that originally described by Gahmberg and Hakomori (136) and is outlined in the flow chart shown in Figure 5. The cells were grown on Blake bottles until almost confluent, washed thoroughly and then treated with 2×10^{-5}M sodium borohydride in order to reduce the amount of non-specific radioactivity incorporated by the latter treatment with [^{3}H]-sodium borohydride. The monolayer of cells was then incubated with galactose oxidase, a treatment which leads to the oxidation of the primary hydroxyl group on exposed galactosyl groups of glycoproteins and glycolipids.

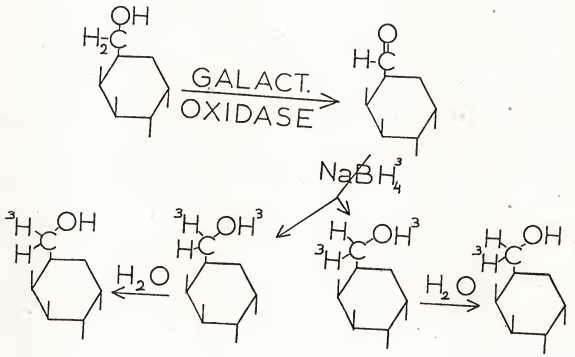
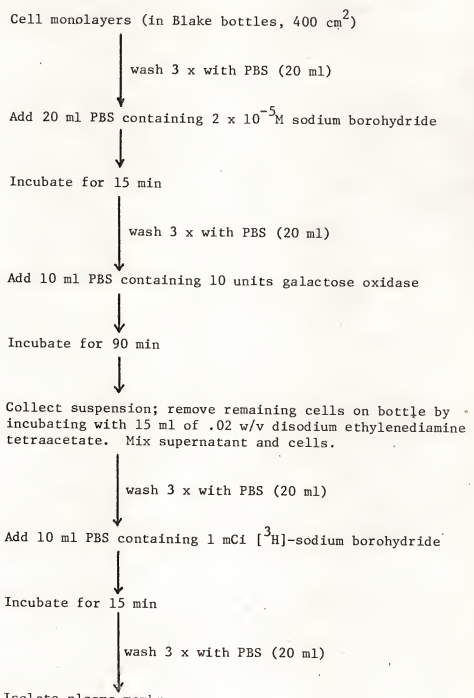


Figure 5: Flow chart demonstrating the procedure for galactose oxidase mediated labeling of cell surfaces.



I found that under these conditions many of the cells detach from the substratum. Therefore, I chose to remove all of the cells using a solution of disodium ethylenediamino tetra acetate (0.02% in PBS). The suspension was then washed with PBS and treated with 1 mCi of [^{3}H]-sodium borohydride in PBS in order to reduce the aldehyde groups on the C-6 position of the galactosialdose. Finally plasma membranes were prepared by the method of Brunette and Till (145).

Reduction of Schiff's bases formed between surface amino groups and pyridoxal phosphate

This reaction is based on the reaction between surface amino groups and pyridoxal phosphate, and the subsequent reduction of the Schiff bases with [^{3}H]-sodium borohydride (135). This reaction is shown in Figure 6a.

The procedure is outlined in the flow chart shown in Figure 6b. After thoroughly washing the monolayer, the cells were covered with PBS containing 0.002 mM pyridoxal phosphate, and incubated for 15 min. The monolayer was then washed with PBS and finally covered with PBS containing 1 mCi [^{3}H]-sodium borohydride. After 15 min the cells were washed with PBS and plasma membranes isolated by the procedure of Barland and Schroeder (143).

Radioactivity Counting Procedures

Counting method of ß-emitters

Samples were counted in the soluene-Triton X-100 3:1 by volume) scintillation fluid described by Turner (143), using a Beckman LS-300 or Nuclear Chicago Isocap scintillation spectrometer. In single label experiments ^{14}C was counted with about 95% efficiency, and ^{3}H with 40%

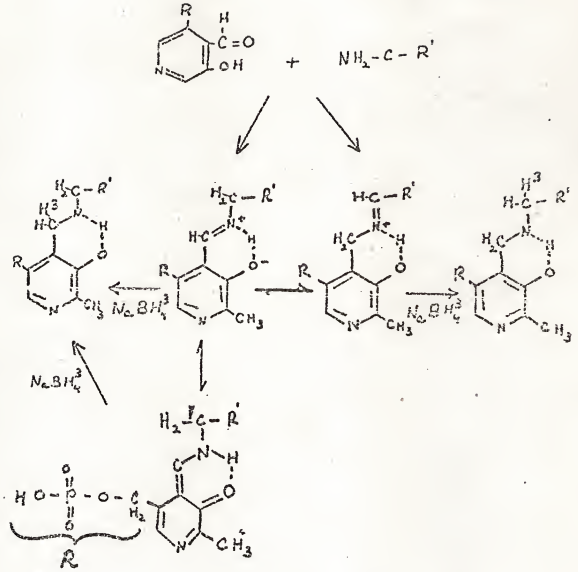
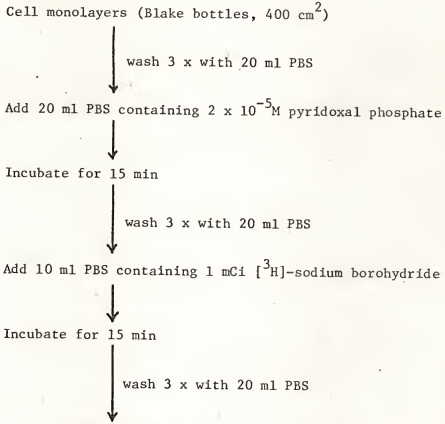


Figure 6a. Reactions involved in the pyridoxal phosphate mediated labeling of cell surfaces.

Figure 6b. Flow chart demonstrating the pyridoxal phosphate mediated labeling of cell surfaces.



efficiency. Weighed samples of ^{14}C and ^{3}H labeled toluene were used as standards. The counting efficiencies of new batches of scintillation fluid were measured regularly throughout this study.

In double label experiments with ^{3}H and ^{14}C, the channels were so adjusted that ^{3}H could be counted maximally, with no overlap into the ^{14}C channel. Depending upon the instrument, the overlap of ^{14}C into the ^{3}H channel was held constant between 40 and 90%. The possibility that quenching might alter the overlap was checked in two ways. (a) The external standard to channel ratio was measured, (b) single label, usually ^{14}C, samples were processed and counted under conditions identical to those used for double labeling experiments. For example in certain cases [^{14}C]-leucine labeled membrane proteins were separated by SDS-polyacrylamide gel electrophoresis. The gels were then sliced and the radioactivity of each slice was measured. By these techniques it was demonstrated that the overlap of ^{14}C into the ^{3}H channel was constant along such a gel and that quench conditions were not variable.

Counting Method of γ-Emitter

The membrane proteins were labeled with ^{125}I, and separated on SDS-polyacrylamide gels. These gels were sliced and each slice was placed in a 100 x 13 mm test tube and the radioactivity was determined in a Packard Autogamma counter, adjusted for ^{125}I counting.

Calculation of ^{3}H and ^{14}C Radioactivity in Double Labeling Experiments

A detailed description of the program used to compute these values on the Wang calculator is given in Appendix A of this dissertation. It is based on the following equations.

Let: for ^{14}C

- blank	B
- counts in standard	C
- counts in ^{3}H-channel	C'
- counts in sample	C_x
- DPM in standard	D
- DPM in sample	D_x
- efficiency $(\frac{C-B}{D})$	E

for ^{3}H

- blank	B*
-counts in standard	C*
- counts in sample	C^*_x
- DPM in standard	D*
- DPM in sample	D^*_x
- efficiency $(\frac{C^*-B^*}{D^*})$	E*
Percentage overlap into ^{3}H	%

Equations:

$$E = \frac{C-B}{D} \qquad E^* = \frac{C^*-B^*}{D^*}$$

$$\% = \frac{(\frac{C'-B^*}{D})}{D_x}$$

$$D_x = \frac{C_x-B}{E}$$

$$D^*_x = \frac{C^*_x-B^*-\%}{E^*}$$

Isolation of Plasma Membranes

Two methods were favored because each of them gave a high yield of plasma membranes by a relatively simple methodology.

Method of Barland and Schroeder

A modification of the original method of Barland and Schroeder (143) was employed in order to reduce the amount of contamination by nuclei. Figure 7 shows a flow chart of the procedure followed. The almost confluent monolayer of cells was treated with a solution of zinc chloride in aqueous dimethylsulfoxide. This fixes and swells the cells. A solution of fluorescein mercuric acetate was then added and the vessels were shaken on ice for periods up to 45 min. Under these conditions the upper surface of the cells (A) are stripped away in sheets from the monolayer, as illustrated below, while the lower surface (B) remains attached to the substratum.

The appearance of these membrane sheets can be followed under a phase microscope. The membrane fragments are then further purified by density gradient centrifugation, boiled in 2% SDS in 0.2M Tris-HCl pH 8.3, dialyzed overnight against 0.1% SDS, 0.1% β-mercaptoethanol in 0.2M Tris-HCl pH 8.3 and analyzed on SDS-polyacrylamide gels.

Method of Brunette and Till

Because the previous method of membrane preparation is in a sense selective for the upper surface of the cell, the method of Brunette and Till (145) was also used. This method gives a significantly lower yield than the previous method. The flow chart in Figure 8 shows the procedure followed in the Brunette and Till method of membrane isolation. The cells were removed from the substratum by incubation with ethylene

Figure 7: Flow chart demonstrating the procedure followed in the isolation of plasma membranes, by the method of Barland and Schroeder.

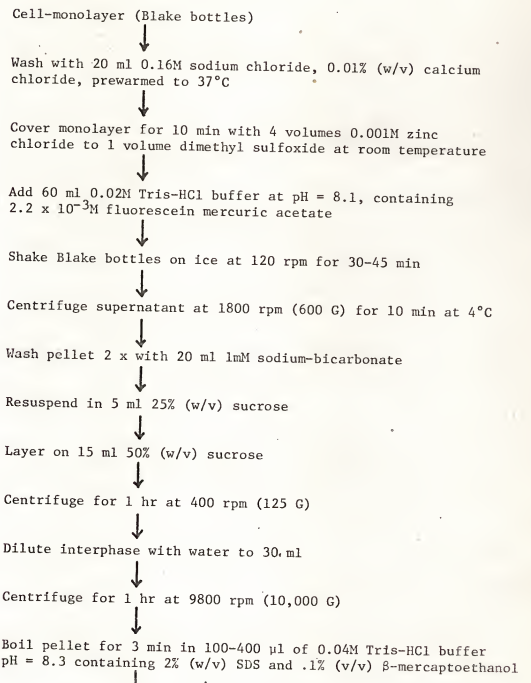


Figure 8: Flow chart demonstrating the procedure followed in the isolation of plasma membranes, by the method of Brunette and Till.

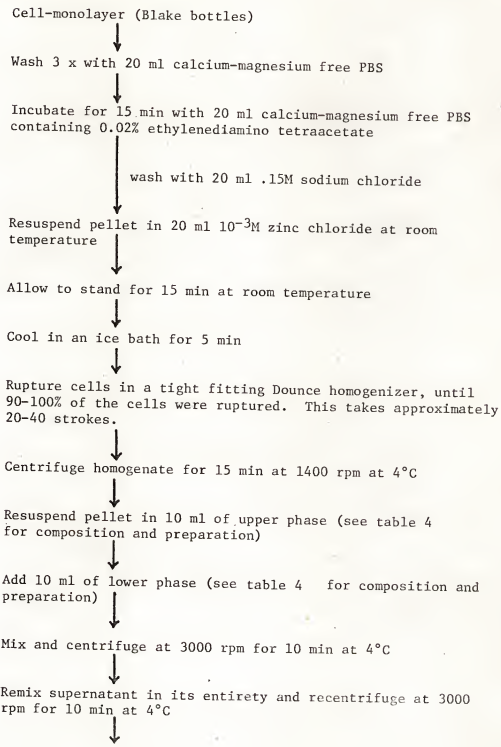


Figure 8 Continued

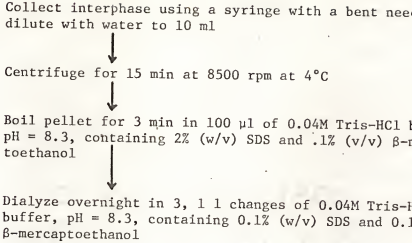


TABLE 4. Preparation and composition of the two phases used in the Brunette and Till method of membrane preparation

Mix:

- 200 g of 20% (w/w) Dextran 500 in water
- 103 g of 30% (w/w) polyethylene glycol in water
- 99 ml of double distilled water
- 333 ml of .22M phosphate buffer, pH = 6.5
- 80 ml of 10^{-2}M zinc chloride

This is mixed thoroughly and allowed to settle overnight in a separatory funnel. The two phases can be collected and are stored at 4°C until used.

diaminotetraacetate. They were then washed with isotonic sodium chloride in water and the pellet of cells resuspended in hypotonic zinc chloride for 15 min at room temperature and subsequently for 5 min in an ice-bath. At this stage the cells were swollen to several times their normal size. They were ruptured in a Dounce homogenizer with a tight fitting pestle. The extent of cell breakage could be followed by phase contrast microscopy. The plasma membranes were purified by centrifugation in a two phase polymer system. The preparation and composition of these phases are given in Table 4.

The material which collected at the interphase between the two phases was collected, resuspended in water and centrifuged. The pellet was dissolved in SDS solution, and analyzed on SDS-polyacrylamide gels.

Determination of Protein Concentrations

All protein determinations, except for those samples containing β-mercaptoethanol, were performed by the procedure of Lowry et al. (146). There was no difference in standard curve for samples without SDS or those with 0.1% (w/v) SDS, see Figure 9. When the SDS concentration was 3% (w/v) there was a slight, but definite, difference in the standard curve. When β-mercaptoethanol was present, the modified procedure of Ross and Schatz (147) was used, since thiol groups interfere strongly with the standard Lowry assay. In the procedure of Ross and Schatz the interfering thiol groups were precipitated prior to color development using iodoacetic acid.

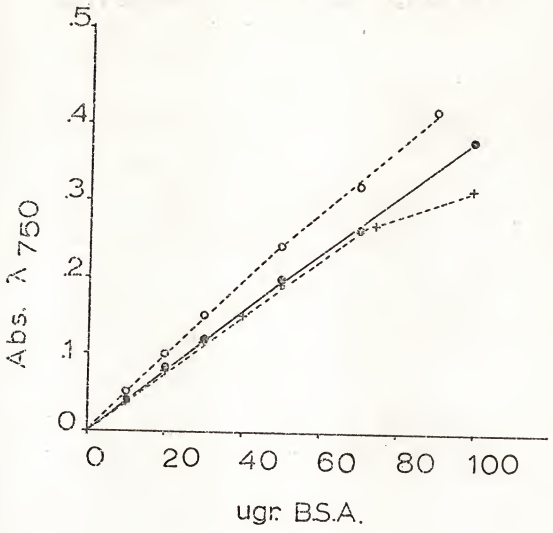


Figure 9. Standard curves for the determination of protein concentration.

0 Determined by the method of Lowry et al.

● Determined by the method of Lowry et al., in the presence of 3% (w/v) SDS.

X Determined by the modified procedure of Ross and Schatz.

Bovine serum albumin was used as standard.

Determination of Agglutination by Wheat Germ Agglutinin and Concanavalin A

In all the experiments in which lectin mediated cell-agglutination was measured, the cells were grown in 100 cm^2 Falcon dishes, Bioquest, Cockeysville, MD, on medium defined in the section on cell maintenance.

The cells were plated at a density of approximately 8 x 10^5 cells per plate, and grown for at least 48 h to between 60 and 80% confluency. The cells were washed 3 times with 5 ml calcium magnesium free phosphate balanced saline, CMF-PBS, and subsequently 3 times with CMF-PBS containing 0.02% EDTA, and incubated for 15 min at 37°C in 5 ml of CMF-PBS containing 0.02% EDTA. The cells were washed off the plate, centrifuged at 700 rpm for 3 min, washed twice with 5 ml PBS, and resuspended in 1.0 ml PBS. Cells from 5 dishes were pooled for each measurement. 0.2 ml of this cell suspension containing about 1.0 x 10^6 cells/cm^2 was placed in a spot plate maintained at 22°C. The desired concentration of lectin was then added and the cells were incubated for 15 min. The total number of single cells, and of cells in aggregates of 3 or more were counted for various regions of the microscope slide. From this the average percent agglutination was determined, as the total number of cells in aggregates, divided by the total number of cells, both free and in aggregates.

Drugs were added, or removed from the medium at intervals and concentrations indicated for each experiment.

Determination of Intracellular Concentrations of 3':5'-Cyclic Adenosine Monophosphate

The 3':5'-cyclic AMP concentration in the cells was estimated using a modification of the protein binding assay of Gilman (148). A special kit containing the appropriate reagents was purchased from

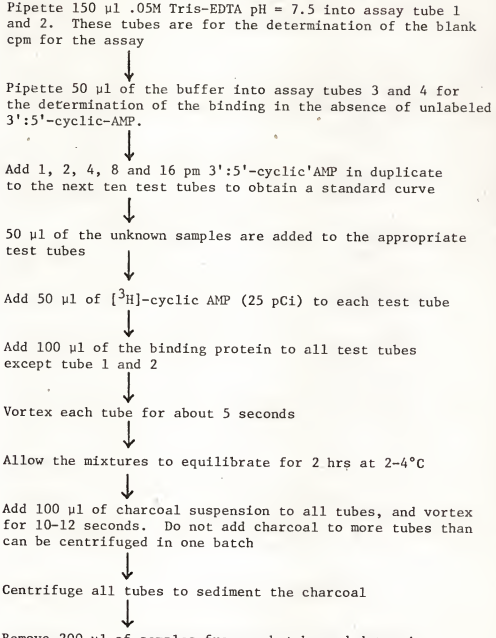
Amersham-Searle, Arlington Heights, Ill. The assay is based on the competition for a binding site on a 3':5'-cyclic-AMP binding protein from bovine muscle between a known amount of [^{3}H]-3':5'-cyclic-AMP added during the assay, and the 3':5'-cyclic-AMP in the sample. The unbound [^{3}H]-3':5'-cyclic-AMP is removed with charcoal and the amount of [^{3}H]-3':5'-cyclic-AMP bound to the protein is determined by liquid scintillation counting. This is, of course, inversly proportional to the amount of 3':5'-cyclic-AMP in the cell. The assay is most sensitive between .5 and 8 pmoles 3':5'-cyclic-AMP in the sample. 8×10^5 cells were inoculated into 100 cm^2 Falcon dishes containing 15 ml medium, as defined in the section on cell maintenance. By allowing the cells to grow for different lengths of time, the 3':5'-cyclic-AMP concentrations could be determined for cell cultures at different cell densities.

After the cells had reached the desired density, the cells were washed thoroughly with PB and solubilized by incubation with 5 ml .1N sodium hydroxide for 5 min. This solution was transferred to a test tube, and 5 ml 5% trichloroacetic acid was added. The mixture was allowed to stand for 30 min at 4°C. This precipitated material was centrifuged, using the maximum setting of a clinical table-top centrifuge. The amount of protein in the precipitate was determined and used as an indirect measure of cell density. The supernatant was mixed 5 x with 10 ml water saturated ether and allowed to reseparate. The residual ether was removed by passing a nitrogen stream through the solution. The solution was lyopholyzed and the sample resuspended in 0.15 ml .05M Tris-EDTA buffer pH = 7.4. The determination of the 3':5'-cyclic-AMP concentration in the samples prepared as described above was performed at 0°C.

The procedure used is outlined in Figure 10. A known amount of [^{3}H]-3':5'-cyclic AMP and binding protein was added to 50 µl of the samples. This mixture was allowed to equilibrate and the excess labeled and unlabeled 3':5'-cyclic AMP was removed with charcoal. The amount of bound [^{3}H]-3':5'-cyclic AMP was determined by liquid scintillation counting. Each determination was performed in duplicate and the average used in the calculations.

To determine the blank value, tubes 1 and 2, which did not contain binding protein, were averaged. The radioactivity bound to binding protein in the absence of unlabeled 3':5'-cyclic AMP (C_o) was determined. Figure 11 shows a typical standard curve obtained by this method.

Figure 10: Experimental procedure used in the determination of 3':5'-cyclic-AMP concentrations.



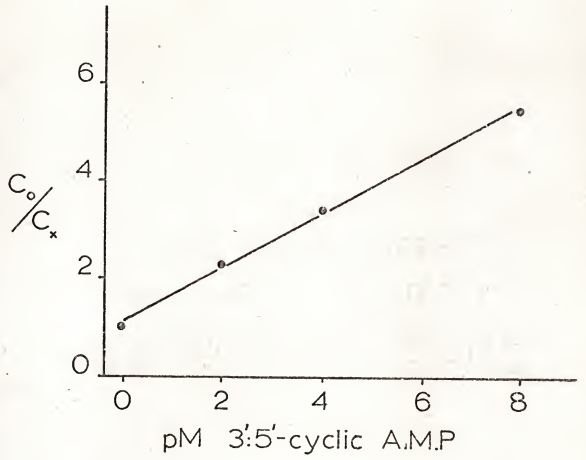


Figure 11. Standard curve for the determination of 3':5'-cyclic AMP concentration.

Co = The radioactivity bound to the binding protein in the absence of unlabeled 3':5'-cyclic-AMP.

Cx = The radioactivity bound to binding proteins in sample number x.

RESULTS

Morphology of the Cell Lines

When normal fibroblasts are transformed either by oncogenic viruses or by carcinogenic agents, they generally lose their elongated shape and assume a more compact morphology, similar to that of epithelial cells. Moreover, they no longer tend to align in parallel arrays, but show a more random distribution on the growth plate. The reverse of these changes were observed when cell lines K-1 and H-7 were grown on medium containing 1mM dB-cAMP. The fibroblast-like line 24-2 was much less affected by the drug, but nevertheless, the cells did appear to be somewhat more elongated. M-7 which closely resembles K-1 and H-7 under normal growth conditions, showed no morphological or visible growth responses to dB-cAMP. Butyric acid, which would arise from any decomposition of dB-cAMP in the growth medium did not have any influence on the morphology of any of the cells when present at a concentration of 1mM. The effects of 3':5'-cyclic AMP on the morphology of the cells were much less pronounced than dB-cAMP. The 3':5'-cyclic AMP diesterase inhibitor Sq 20009 had a similar, though less pronounced effect on line M-7, a line which was morphologically unaffected by dB-cAMP. These results are summarized in Table 5 and Figure 12. Although the untreated cells vary in morphology but do not have significantly different 3':5'-cyclic AMP levels (Table 6), these results suggest that the morphological responsiveness of the CHO cells does relate to a

TABLE 5. Effect of 3':5'-cyclic AMP, dB-c-AMP and Sq 20009 on the morphology of the CHO cell lines

Cell Line	No Addition	10^{-3}M c-AMP	10^{-3}M dB-c-AMP	10^{-4}M Sq 20009
K-1	Irregular	+	+++	±
M-7	Irregular	0	0	+
24-2	Elongated	±	±	±
H-7	Irregular	+	++	±

0 = no elongation; + = little elongation; +++ = pronounced elongation

± = response could not be established unequivocally

Figure 12. The effect of dB-c-AMP or SQ 20009 on the morphology of CHO cells.

The CHO lines K-1-24-2 (1), K-1 (2), H-7 (3) and K-1-M-7 (4) were grown on 10% fetal calf serum containing McCoy's 5A medium (A) in the presence of 10^{-3}M dB-c-AMP (B) or 10^{-4}M SQ 20009 (C). After staining with Toluidine Blue they were photographed on the culture dish.

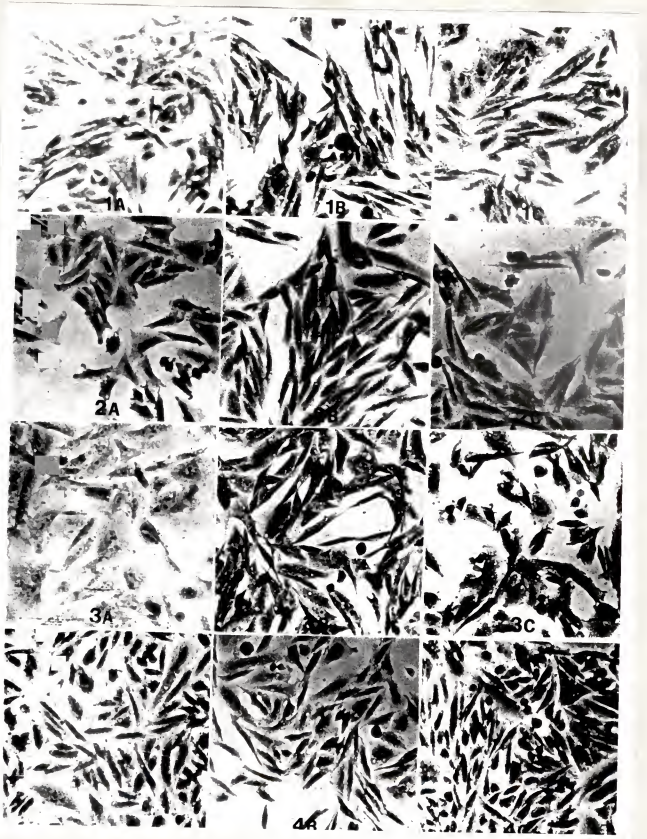

1A
1B
2A
3A
3C
4A

TABLE 6. The intracellular 3':5'-cyclic AMP levels in the CHO cell lines

Cell Line	Number of Cells (x 10^6)	µg Protein/Dish	pM cAMP/mg Protein
H-7	12.4	2688	1.79
	15.4	3360	9.0
K-1	12.1	2640	2.4
	18.2	3960	11.4
K-1-M-7	12.7	2760	3.1
	16.0	3480	12.4
K-1-24-2	12.8	2784	4.8
	18.7	4080	6.4

change in internal 3':5'-cyclic-AMP concentration within the cell. As discussed in the introduction, the lack of potency of 3':5'-cyclic-AMP itself possibly relates to either its failure to penetrate the cells (15-17) or to its greater instability (16,17) as compared to the dibutyryl analogue. The above results are in general agreement with those of others using a wide number of transformed fibroblast lines, as discussed in the introduction.

The Effect of dB-cAMP on the Growth Rate of the Cells and on the Uptake and Incorporation of [^{3}H]-Thymidine

A number of other studies have revealed that dB-cAMP reduces the growth rate of transformed fibroblasts grown in culture (32-34). In the cells used in this study this was not the case. That all cells do continue through the cell cycle in the presence of dB-cAMP was shown by low serum synchronization of the cells. Cell cultures at approximately 30% confluency were transferred to 0.5% (v/v) fetal calf serum containing medium for up to 36 h. Serum was then added to a final concentration of 10% (v/v). Figure 13a shows that all cells do indeed divide synchronously after approximately 11 h. In the experiment shown in Figure 13b, I examined the growth rate of the four CHO cell lines in the presence and absence of 1mM dB-cAMP. Cells (2×10^5) were transferred to 25 cm Falcon dishes containing 5 ml of medium. After allowing the cells 12 h to attach to the substratum, cells from duplicate plates were removed by trypsinization at 12 h intervals, and counted using a Coulter counter.

It can be seen clearly from Figure 13b that the growth rates of the cell lines were not affected by dB-cAMP in the medium. In each case the generation time was around 16 h whether or not the drug was present.

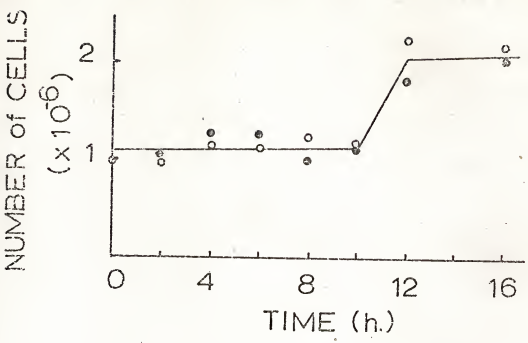


Figure 13a. The effect of dB-cAMP on the synchronization of CHO-H-7

Highly confluent cells were subcultured and grown for 22 h in the presence or absence of 1 mM dB-cAMP. They were then transferred to medium containing 0.5% (v/v) fetal calf serum for 16 h. dB-cAMP was present in the same cultures as in the previous incubation. At 2 h intervals duplicate cultures were used for both sets of cells to determine cell number. The cells were removed from the plate by a 1/2 min incubation with 0.25% (v/v) trypsin-PB and counted with a Coulter counter. No dB-cAMP (O), 1 mM dB-cAMP (O).

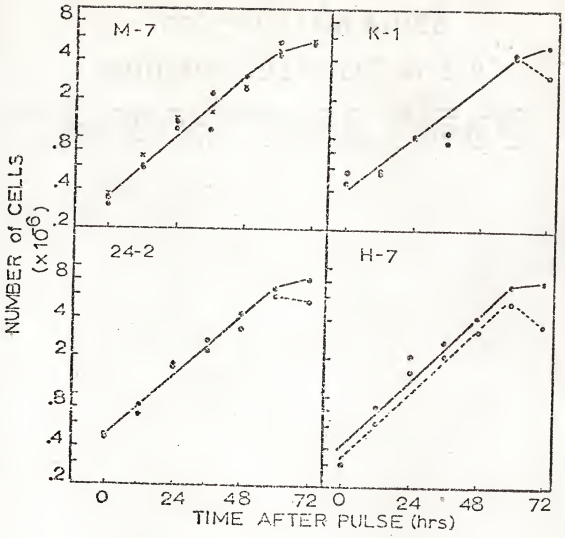


Figure 13b. The effect of dB-cAMP on the growth rate of the CHO cells.

The Cells were grown in the presence or absence of 1mM dB-cAMP. At 12 h intervals cells were removed from a flask by trypsin and counted using a Coulter counter.

M-7; control (●); + 1mM dB-cAMP (○) and + 0.1mM Sq 20009 (x)

K-1; control (●); + 1mM dB-cAMP (○)

24-2; control (●); + 1mM dB-cAMP (○)

H-7; control (●); + 1mM dB-cAMP (○)

The growth rate of the treated cells did appear to fall away in the last 12 h of the experiment, so that the final cell density was reduced by about 30% compared with the controls. However, it should be emphasized that the medium was not changed in these experiments, and might be more "depleted" in one set of cells than in the other. The final cell density achieved by the untreated CHO cells in an experiment of this kind was 6×10^6 cells/plate. Addition of new medium does not increase this value significantly since mitotic cells are shed from the monolayer and remain in suspension. However, it seems clear from these experiments that dB-cAMP does not reduce growth rate significantly nor promote a condition that might be regarded as "contact inhibition of growth."

In the same experiment in which I measured cell number, I also measured the ability of the cells to incorporate [^{3}H]-thymidine into trichloracetic acid-soluble and -insoluble fractions. Every 10-12 h a "pulse" of radioactivity (2.5 μCi/ml [^{3}H]-thymidine) was administered to duplicate flasks of cells. The cells were then trypsinized, washed with PBS and resuspended in 5 ml PBS. One ml of the cell solution was used for the counting of cells in a Coulter counter while the remainder was centrifuged. The pellet was resuspended in 5 ml cold 10% (w/v) trichloracetic acid-water and allowed to stand at 4°C overnight. The suspension was centrifuged and the radioactive content of the supernatant fraction and pellet was measured.

Figure 14 shows the amount of ^{3}H recovered from the cells. It is clear that the presence of dB-cAMP significantly increases the total uptake of thymidine by the cells. Therefore, a comparison of the amount of radioactivity incorporated into the cells is not a useful measure of

Figure 14. The effect of dB-cAMP on the thymidine uptake in CHO cells.

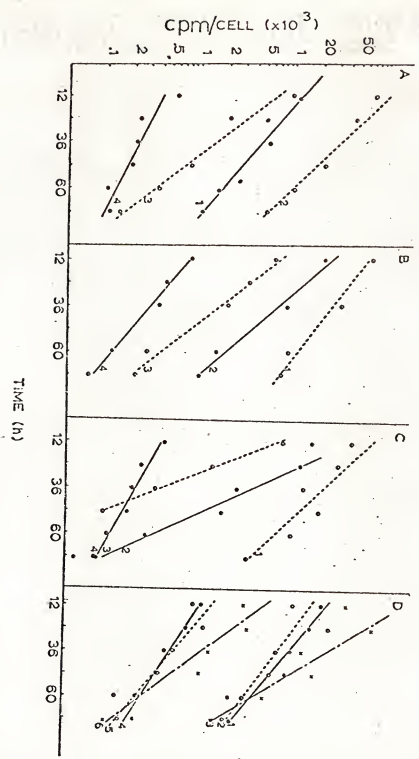
At 12 h intervals cells in log phase of growth were pulsed with 12.5 μCi [^{3}H]-thymidine for 2 h. The cells were extensively washed and then disrupted in 5% trichloroacetic acid. Incorporation of ^{3}H into trichloroacetic acid insoluble material was used as a measure of DNA synthesis, while the radioactivity in the supernatant was used as a measure of the uptake of thymidine.

A H-7 Incorporation into DNA in the presence (2) or absence (1) of 1 mM dB-cAMP. Uptake in the presence (3) or absence (4) of 1 mM dB-cAMP.

B K-1 Incorporation into DNA in the presence (1) or absence (2) of 1 mM dB-cAMP. Uptake in the presence (3) or absence (4) of 1 mM dB-cAMP.

C 24-2 Incorporation into DNA in the presence (1) or absence (2) of 1 mM dB-cAMP. Uptake in the presence (3) or absence (4) of 1 mM dB-cAMP.

D M-7 Incorporation into DNA in the presence (3) or absence (1) of 1 mM dB-cAMP or in the presence of 0.1 mM Sq 20009 (2). Uptake into DNA in the presence (6) or absence (4) of 1 mM dB-cAMP or in the presence of 0.1 mM Sq 20009 (5).


cpm/CELL (x10 3)
.1
.2
.5
1
2
5
10
20
50
A
B
C
D
12
36
60
TIME (h)

the relative amounts of DNA synthesized. Secondly, Figure 14 indicates that as cell density increases the amount of thymidine incorporated declines even though the rate of cell division had not fallen (Figure 13b). Clearly, great care has to be taken in interpreting experiments in which rates of DNA synthesis are measured by determining radioactive thymidine incorporation by whole cells.

Comparison of Plasma Membrane Polypeptides of the Different Cell Lines

A comparative study of the plasma membrane of the different cell lines revealed no major differences in the polypeptides which are present as analyzed by gel electrophoresis in SDS-containing polyacrylamide gels. In these experiments, the plasma membrane polypeptides were isolated according to the procedure of Barland and Schroeder (143). The method is highly reproducible, and the isolated plasma membrane fraction can be stored at -4°C for extended periods of time. From Figure 15 it can be seen that no marked differences were observable between two preparations which had been isolated as much as three months apart, and subjected to electrophoresis on the same polyacrylamide gel.

Figures 16, 17a and 18 show gels of plasma membrane preparations stained with Coomassie Blue. Three concentrations of polyacrylamide were employed in order to allow a more complete resolution of the component polypeptides. In Figure 17a (7.5% (w/v) polyacrylamide) the migration of the polypeptides has been compared with four standards of known molecular weight. Figure 17b also shows a typical calibration curve obtained for this gel concentration. Polypeptides ranged in molecular weight from around 120,000 to less than 15,000. Very little material was located at the very top of the gel suggesting that the solubilization in detergent was complete and that few proteins of

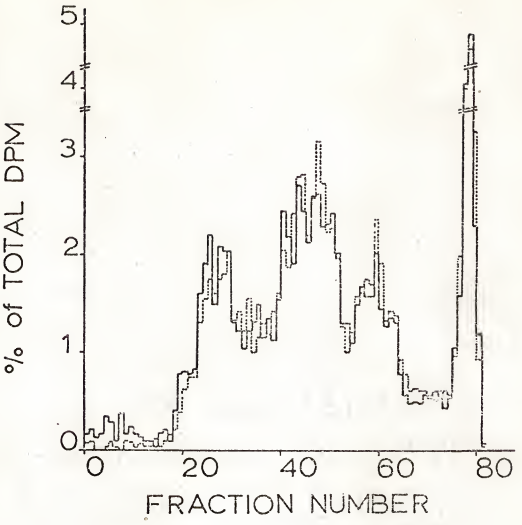


Figure 15. Reproducibility of membrane preparations.

Two different membrane preparations of CHO-K-1 labeled with either ^{3}H or ^{14}C, and prepared approximately 3 months apart, were co-electrophoresed on 7.5% (w/v) polyacrylamide gels. The gels were sliced and radioactivity in each slice was determined. (——L-[^{3}H]-leucine newer preparation; ------ L-[^{14}C]-leucine older preparation).

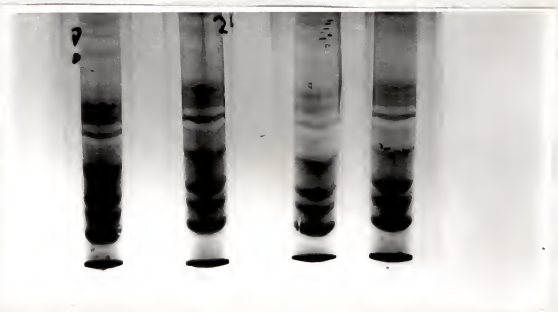


Figure 16. Coomassie blue staining pattern after polyacrylamide gel electrophoresis of the plasma membrane polypeptides from CHO lines K-1 (a), 24-2 (b), H-7 (c) and M-7 (d). Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 5% (w/v) polyacrylamide gels.

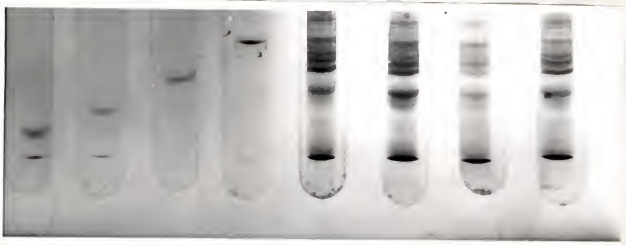


Figure 17a. Coomassie blue staining pattern after polyacrylamide gel electrophoresis of the plasma membrane polypeptides from CHO lines K-1 (a), 24-2 (b), H-7 (c) and M-7 (d). Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 7.5% (w/v) polyacrylamide gels.

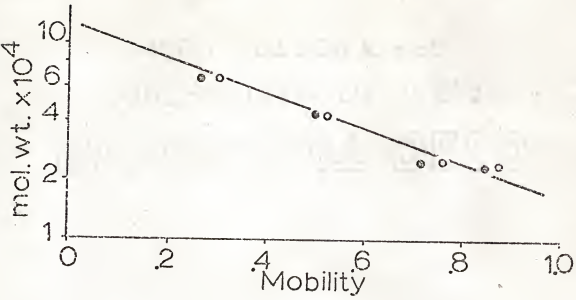


Figure 17b. Standard curve for molecular weight determinations using the 7.5% (w/v) polyacrylamide gel system. Standards are BSA (MW 68,000); ovalbumin (MW 44,000); trypsin (MW 23,800) and chymotrypsinogen (MW 25,000).

Figure 18. Coomassie blue staining pattern after polyacrylamide gel electrophoresis of the plasma membrane polypeptides from CHO lines K-1 (a), 24-2 (b), H-7 (c) and M-7 (d). Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 10% (w/v) polyacrylamide gels.

high molecular weight analogous to spectrin of erythrocytes (149) were present. The sharp band at the bottom of the gel corresponded with the dye front in the 7.5% (w/v) and 5% (w/v) gels. It probably represents polypeptides of relatively low molecular weight which can be resolved only in the 10% (w/v) gel system (Figure 18). Note that in Figure 18 when 10% (w/v) polyacrylamide concentrations were used, this band is not present. Five major polypeptide species (molecular weights 38,000, 47,500, 54,000, 58,000, 68,000) can be identified in each CHO cell line and are indicated by arrows on Figure 17a. No qualitative differences appear to distinguish the preparations from each other at any of the gel concentrations employed. Similar numbers of bands with approximately similar staining intensities can be observed.

In order to make quantitative comparisons in the polypeptide compositions of the plasma membranes derived from the different cell lines I grew the cells to 90% confluency on radioactive L-leucine as described in the Materials and Methods section. In this experiment, the line CHO-K-1 was provided with L-[^{14}C]-leucine (0.16 μCi/ml medium) for 48 h, and the other lines with L-[^{3}H]-leucine (0.9 μCi/ml medium). Portions of labeled membrane material were electrophoresed in 7.5% (w/v) gels (Figure 19). Clearly when the samples were run separately it was impossible to make accurate quantitative comparisons due to slight variations in the gel-slicing procedure. In order to overcome this, contrastingly labeled samples were mixed and co-electrophoresed. Figure 20 shows the result of one such experiment using 10% (w/v) polyacrylamide gels. In this experiment, the line K-1 labeled with

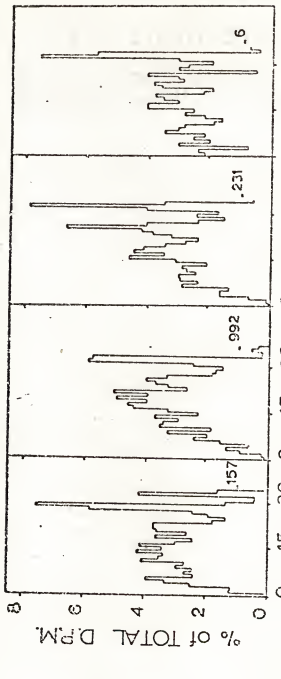


Figure 19. Profile of radioactivity after polyacrylamide gel electrophoresis of the plasma membrane polypeptides from CHO lines M-7 (a), 24-2 (b), H-7 (c) labeled with 10 μCi [^{3}H]-leucine and K-1 (d) labeled with 5 μCi [^{14}C]-leucine. Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 7.5% (w/w) polyacrylamide gels.

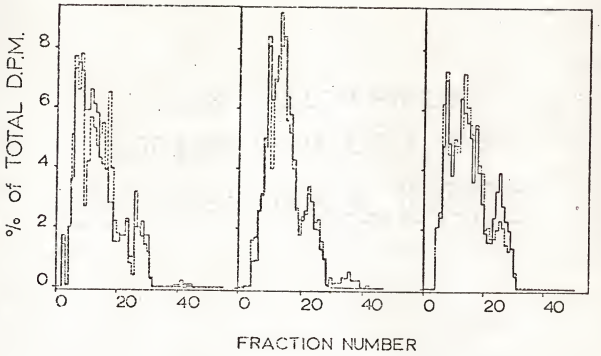


Figure 20. Profile of radioactivity after polyacrylamide gel electrophoresis of the plasma membrane polypeptides from CHO line K-1 labeled with 5 μCi [^{14}C]-leucine (———) mixed with membrane polypeptides from H-7 (a), M-7 (b) and 24-2 (c) labeled with 10 μCi [^{3}H]-leucine (-----). Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 10% (w/v) polyacrylamide gels.

^{14}C provided the basis for comparisons. From this experiment, it appears that while M-7 and 24-2 show great similarity to the K-1 strain, the H-7 line shows some quantitative differences from the others. These results are not entirely unexpected, since M-7 and 24-2 were immediate derivatives of K-1 while H-7 originated from a different laboratory. Clearly although the same polypeptide species were present in H-7 as the other lines, these were not present in exactly similar proportions.

Effects of dB-cAMP on the Plasma Membrane Polypeptide Composition

In these experiments comparisons were made between the treated cells and their untreated counterparts by growing them on contrastingly labeled L-leucine, isolating plasma membranes from each, mixing the preparations and subjecting the mixture to polyacrylamide gel electrophoresis. The results of a typical series of experiments are shown in Figure 21. In these, the treated cells had been grown on 1mM dB-cAMP in the presence of 10 μCi of [^{3}H]-L-leucine for 2 days, while the normal cells had been provided with 5 μCi of [^{14}C]-L-leucine.

This growth period constituted approximately 3 to 4 cell generations and was probably sufficient to allow all of the membrane proteins to reach similar specific radioactivities. Two different polyacrylamide preparations (7.5% and 10%) were employed in order to resolve more completely, the different size classes of polypeptide. It is clear from Figure 21 and Figure 22 that no major qualitative or quantitative differences could be observed between the controls and the treated cells. Similar components were present in almost identical proportions. Nevertheless, SDS-electrophoresis separates proteins by size and we

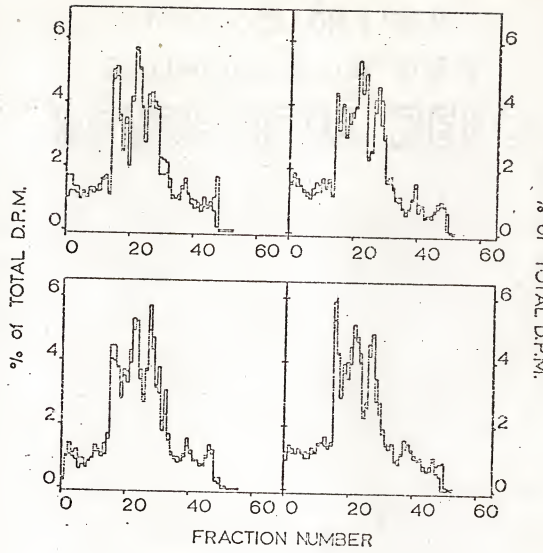


Figure 21. Profile of radioactivity after polyacrylamide gel electrophoresis of the plasma membrane polypeptides of the CHO lines grown in the presence (———) or absence (-----) of 1 mM dB-cAMP. Lines H-7 (a) and 24-2 (c) were labeled with 10 μCi [^{3}H]-leucine in the absence and with 5 μCi [^{14}C]-leucine in the presence of dB-cAMP. Lines M-7 (b) and K-1 (d) were labeled with 5 μCi [^{14}C]-leucine in the absence and with 10 μCi [^{3}H]-leicine in the presence of dB-cAMP.

Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 10% (w/v) polyacrylamide gels.

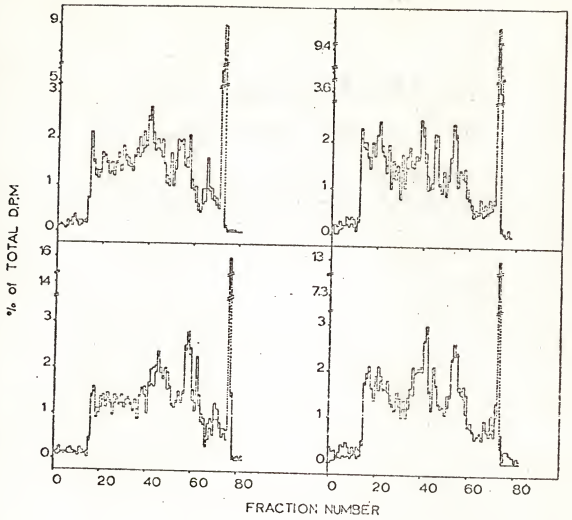


Figure 22. Profile of radioactivity after polyacrylamide gel electrophoresis of the plasma membrane polypeptides of the CHO lines grown in the presence (———) or absence (-----) of 1mM dB-cAMP. Lines H-7 (a) and 24-2 (c) were labeled with 10 μCi [^{3}H]-leucine in the absence and with 5 μCi [^{14}C]-leucine in the presence of dB-cAMP. Lines M-7 (b) and K-1 (d) were labeled with 5 μCi [^{14}C]-leucine in the absence and with 10 μCi [^{3}H]-leucine in the presence of dB-cAMP.

Membranes were prepared by the method of Barland and Schroeder. Electrophoresis was carried out in 7.5% (w/v) polyacrylamide gels.

cannot rule out the possibility that certain proteins have not become modified by phosphorylation or other types of substitution reactions during the course of treatment with dB-cAMP.

Lactoperoxidase mediated iodination of the cell surface of cells grown in the presence or absence of dB-cAMP

Because the loss in agglutinability of the CHO cells induced by dB-cAMP, which will be discussed later, might be due to a conformational rearrangement of the plasma membrane which restricts the movement of receptor molecules, I used the lactoperoxidase ^{125}I-labeling method to tag proteins exposed at the cell surface after growth in presence or absence of the nucleotide. Plasma membranes were prepared by the method of Barland and Schroeder. The radioactive polypeptides of the plasma membrane were then separated by electrophoresis in SDS-polyacrylamide gels. Figure 23 shows the results achieved using a 7.5% (w/v) polyacrylamide stacking gel and a 10% (w/v) polyacrylamide running gel (7.5/10% w/v gel). In neither of the cell lines which respond to dB-cAMP, i.e. H-7 (Figure 24) or 24-2 (Figure 25) was there any detectable change in the pattern of radioactivity along the gels. Moreover the profiles of incorporated radioactivity of the two were indistinguishable.

However, since much of the radioactivity was concentrated at the top of the stacking gel, at the interphase between the stacking and running gels and at the dye front, we used a number of other electrophoretic separation conditions. Figures 24-27 show the results of these studies for lines H-7, 24-2, M-7 and K-1 respectively. Probably the most complete separation was achieved using a 7.5% (w/v) running gel in conjunction with a 5% (w/v) stacking gel. For each line studied a very high molecular weight component was again located at the very top of

Figure 23. Iodinatable surface polypeptides of different CHO cell lines grown in the presence or absence of 1mM dB-cAMP. Cells were labeled using the lactoperoxidase technique, membranes prepared by the method of Barland and Schroeder, and polypeptides analyzed on SDS-polyacrylamide gels consisting of a 7.5% (w/v) stacking gels and a 10% (w/v) running gel. The gels were sliced and the radioactive content of each slice was determined.

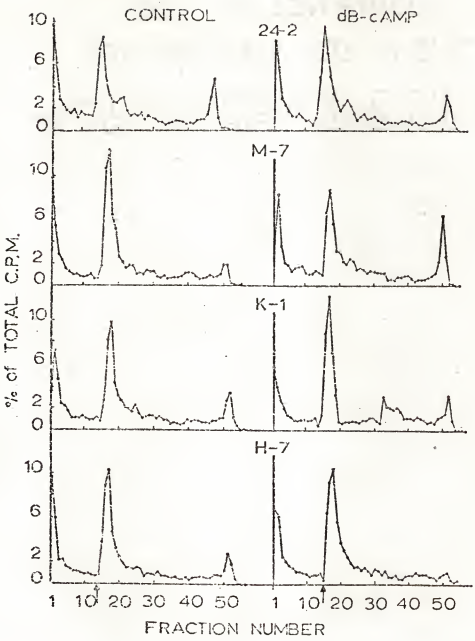

CONTROL
dB-cAMP
24-2
M-7
K-1
H-7
% of TOTAL C.P.M.
10
6
2
0
1 10 20 30 40 50
FRACTION NUMBER

Figure 24. Iodinatable surface polypeptides of CHO-H-7 grown in the presence or absence of dB-cAMP. Cells were labeled using the lactoperoxidase ^{125}I technique. Membranes were prepared by the method of Barland and Schroeder and analyzed on three gel systems:

Top - a gel consisting of a 7.5% (w/v) stacking gel and a 10% (w/v) running gel.

Middle - a gel consisting of a 5% (w/v) stacking gel and a 7.5% (w/v) running gel. In these graphs molecular weight markers are indicated by arrows above the graph.

Bottom - a gel consisting of a 3.75% (w/v) stacking gel and a 7.5% running gel.

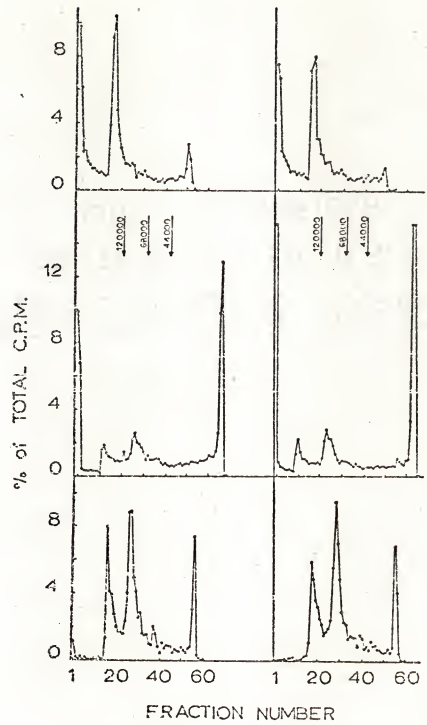

8
4
0
12
8
4
0
8
4
0
120000
68000
41000
120000
68000
41000
% of TOTAL C.P.M.
1
20
40
60
1
20
40
60
FRACTION NUMBER

Figure 25. Iodinatable surface polypeptides of CHO-K-1-24-2 grown in the presence or absence of 1mM dB-cAMP. Cells were labeled using the lactoperoxidase ^{125}I technique. Membranes were prepared by the method of Barland and Schroeder and analyzed on three gel systems:

Top - a gel consisting of a 7.5% (w/v) stacking gel and a 10% (w/v) running gel.

Middle - a gel consisting of a 5% (w/v) stacking gel and a 7.5% (w/v) running gel. In these graphs molecular weight markers are indicated by arrows above the graph.

Bottom - a gel consisting of a 3.75% (w/v) stacking gel and a 7.5% running gel.

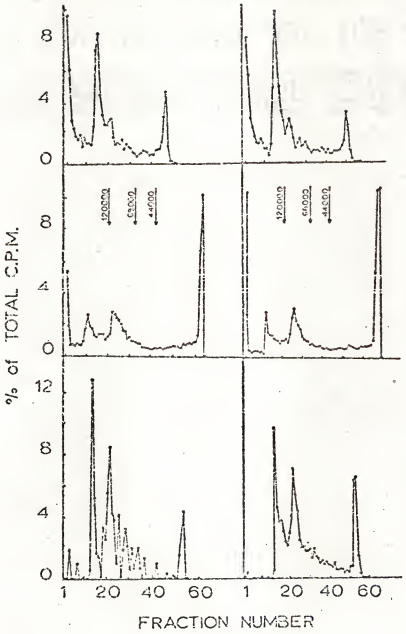

% of TOTAL C.P.M.
8
4
0
8
4
0
12
8
4
0
1
20
40
60
1
20
40
60
FRACTION NUMBER

Figure 26. Iodinatable surface polypeptides of CHO-K-1-M-7 grown in the presence or absence of dB-cAMP. Cells were labeled using the lactoperoxidase ^{125}I technique. Membranes were prepared by the method of Barland and Schroeder and analyzed on three gel systems:

Top - a gel consisting of a 7.5% (w/v) stacking gel and a 10% (w/v) running gel.

Middle - a gel consisting of a 5% (w/v) stacking gel and a 7.5% (w/v) running gel. In these graphs molecular weight markers are indicated by arrows above the graph.

Bottom - a gel consisting of a 3.75% (w/v) stacking gel and a 7.5% running gel.

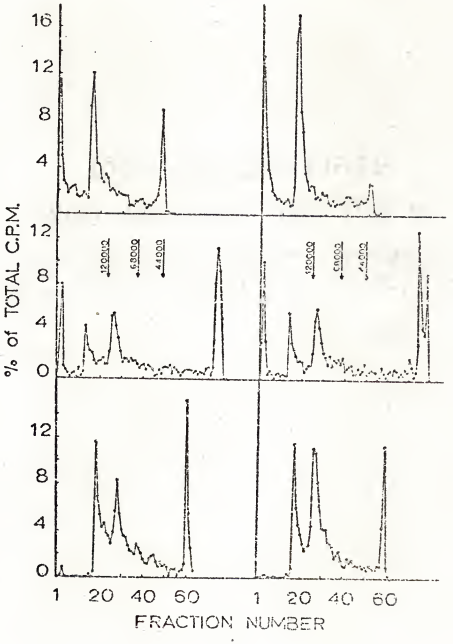
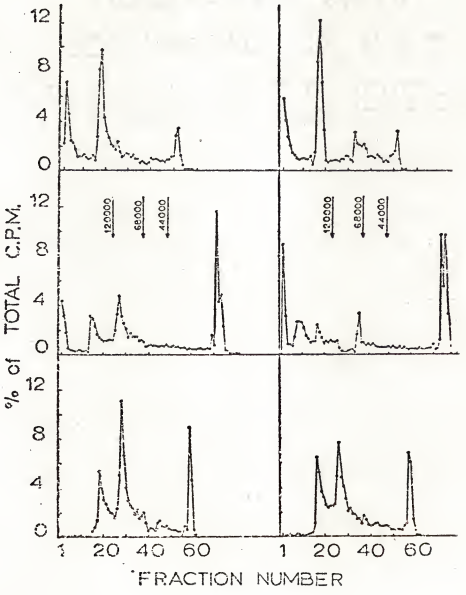

% of TOTAL C.P.M.
16
12
8
4
12
8
4
0
12
8
4
0
1290Da
680Da
443Da
1290Da
680Da
443Da
1
20
40
60
1
20
40
60
FRACTION NUMBER

Figure 27. Iodinatable surface polypeptides of CHO-K-1 grown in the presence or absence of dB-cAMP. Cells were labeled using the lactoperoxidase ^{125}I technique. Membranes were prepared by the method of Barland and Schroeder and analyzed on three gel systems:

Top - a gel consisting of a 7.5% (w/v) stacking gel and a 10% (w/v) running gel.

Middle - a gel consisting of a 5% (w/v) stacking gel and a 7.5% (w/v) running gel. In these graphs molecular weight markers are indicated by arrows above the graph.

Bottom - a gel consisting of a 3.75% (w/v) stacking gel and a 7.5% running gel.


12
8
4
0
120000
68000
44000
100000
68000
44000
% of TOTAL C.P.M.
1
20
40
60
FRACTION NUMBER

the stacking gel. A protein of molecular weight greater than 130,000 was found close to the interphase region. A third band (molecular weight approximately 110,000) was located one-third the way down the gel. Most of the remaining radioactivity collected at the dye front and was in proteins of molecular weight less than 25,000. By using lower concentrations of polyacrylamide in the stacking gel it was possible for the very high molecular weight material to pass through the stacking gel (Figures 24-27) and to band at the top of the running gel.

Examination of these results indicate a) that all of the CHO lines have similar size classes of iodinatable proteins at their surface, b) that as in other cell species (120,129) only a limited number of proteins in the plasma membrane are accessible to the labeling agent, and are sensitive to trypsin (Figure 28), c) that the lines H-7, M-7 and 24-2 show no changes in iodination pattern following growth on dB-cAMP even though H-7 and 24-2 show complete loss of agglutinability under these conditions. We conclude therefore, that there has not been a major rearrangement or masking of surface proteins in response to the dB-cAMP-induced loss of agglutination, d) only line K-1 shows any change in iodination pattern (Figure 26). Here additional bands of molecular weight greater than 130,000 and 72,000 are detectable after growth on dB-cAMP. These changes have not been investigated further since this line does not lose its agglutinability in response to the nucleotide. The significance of the observation is not understood, e) there was no evidence in the treated H-7 line, which both loses its agglutinability and which assumes a fibroblast-like morphology, for the appearance of a new, iodinatable protein of high molecular weight greater than 200,000.

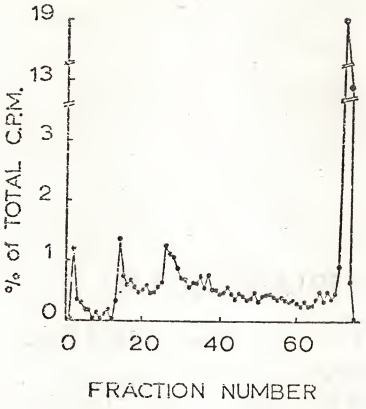


Figure 28. The effect of trypsin treatment on the iodinatable surface polypeptides of CHO-K-1-M-7. The cells were incubated for 1 min with 0.25% trypsin. Subsequently the surface polypeptides of the cells were labeled using the lactoperoxidase technique. Membranes were prepared by the method of Barland and Schroeder, and analyzed on SDS-polyacrylamide gels consisting of a 5% (w/v) stacking gel and a 7.5% (w/v) running gel.

Such a protein has been reported as a persistent component of the plasma membranes of fibroblast-like cells, but is apparently absent in their transformed counterparts (120).

Other methods studied as membrane selective probes

I studied the galactose oxidase and pyridoxal phosphate mediated labeling of the surface components of the plasma membrane. Both these methods finally depend on a reduction by [^{3}H]-sodium borohydride, for the actual introduction of label into the plasma membrane components. We shall show that these methods did not have the specificity required for this study, presumably due to non-specific labeling of protein and other compounds by [^{3}H]-sodium borohydride.

For example, if cells are treated with [^{3}H]-sodium borohydride alone (1mCi, 15 min), their plasma membranes isolated, and polypeptides analyzed by electrophoresis on SDS polyacrylamide gels, a complex pattern of radioactivity was found along the gels (Figure 29) indicating that components of both high and low molecular weight became labeled. If the cells were first treated with unlabeled borohydride in order to react with readily reducible compounds, the pattern of radioactivity was somewhat changed. Most of the label in the higher molecular weight components was lost. A very similar pattern was obtained after CHO cells were incubated with unlabeled sodium borohydride for 15 min and then treated with 10 units of galactose oxidase for 90 min before reduction with 1mCi [^{3}H]-sodium borohydride (5 min). In addition, it can be seen from Table 7 that specific radioactivity of the membranes only increased by a factor of about 2 after the cells had been pretreated with galactose oxidase prior to the reduction with [^{3}H]-sodium borohydride. Figure 29 and 30 show that the pattern of labeling on SDS-polyacrylamide gels is

Figure 29. The non-specific labeling of CHO-H-7 surface polypeptides by [^{3}H]-sodium borohydride. The cells were disrupted in 10% (w/v) trichloroacetic acid, and label incorporated in pellet was analyzed on SDS-polyacrylamide gels consisting of a 5% (w/v) stacking gel and a 7.5% (w/v) running gel. The gels were sliced and the radioactive content of each slice was determined.

Top - cells were incubated for 15 min with 1 mCi [^{3}H]-sodium borohydride, and disrupted in 10% trichloroacetic acid.

Middle - cells were incubated for 15 min with 2 x 10^{-5}M sodium borohydride, after a 90 min incubation with PBS to mimick incubation with galactose oxidase, the cells were incubated for 15 min with 1mCi [^{3}H]-sodium borohydride and membrane proteins analyzed.

Bottom - cells were incubated for 15 min with 2 x 10^{-5}M sodium borohydride, 90 min with 10 units galactose oxidase and finally with 1mCi [^{3}H]-sodium borohydride and membrane proteins analyzed.

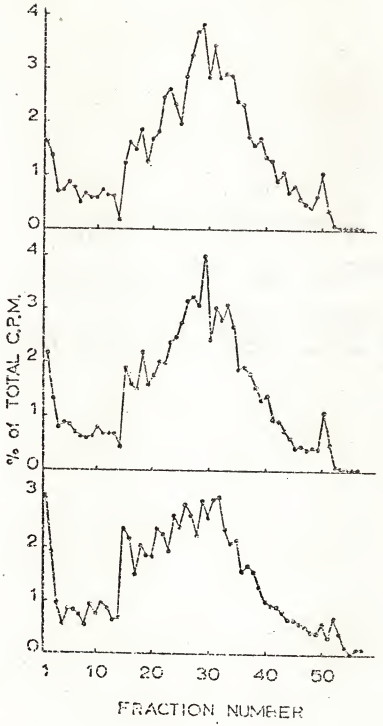

% of TOTAL C.P.M.
FRACTION NUMBER
0
1
2
3
4
10
20
30
40
50

Figure 30. Galactose oxidase mediated labeling of CHO-H-7 surface polypeptides. After specified pretreatments the surface polypeptides were labeled. Membranes were prepared by the method of Brunette and Till, and analyzed on SDS-polyacrylamide gels consisting of a 7.5% (w/v) stacking gel and 10% (w/v) running gel. The gels were sliced and the radioactive content of each slice was determined.

Top - cells were incubated with 2×10^{-5}M sodium borohydride for 15 min, but not incubated with galactose oxidase.

Middle - incubation with sodium borohydride was followed by a 90 min incubation with galactose oxidase.

Bottom - incubation with sodium borohydride was followed by a 2 min incubation with 0.25% trypsin and a subsequent 90 min incubation with galactose oxidase.

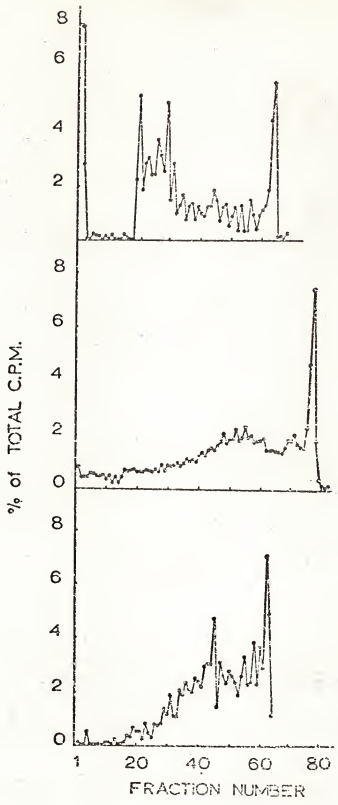

8
6
4
2
8
6
4
2
0
8
6
4
2
0
% of TOTAL C.P.M.
1
20
40
60
80
FRACTION NUMBER

TABLE 7. Specific activity of H-7 membranes prepared by the Brunette and Till method after labeling with the galactose-oxidase-sodium-borotitride technique

Treatment Before Incubation with 1mCi [^{3}H]-sodium borohydride	cpm/µg Protein
Cells were incubated with 2 x 10^{-5} M sodiumborohydride for 15 min	116
Cells were incubated with 2 x 10^{-5} M sodiumborohydride for 15 min and subsequently for 90 min with 10 units of galactose oxidase, after they were cultured with:	
- no addition to the growth medium	229
- 1mM dB-cAMP added to the growth medium for 48 h	283
- 0.1mM Sq 20009 added to the growth medium for 48 h	289
Cells were incubated with 2 x 10^{-5} M sodium borohydride for 15 min, then for 2 min with 0.25% (w/v) trypsin, and a subsequent incubation with 10 units of galactose-oxidase for 90 min	136

not very different either after incubation with galactose oxidase and analysis on two different polyacrylamide gel concentrations. Figure 30, furthermore, shows that only a fraction of labeled molecules can be removed from the cell surface by incubation with trypsin.

Therefore, we felt that the galactose oxidase technique in which ^{3}H is introduced into dialdose groups of glycoproteins following oxidation with galactose oxidase (p. 33 of Materials and Methods), or the technique involving reduction of Schiff's bases to pyridoxal phosphate (Figure 6a), both of which require the use of [^{3}H]-sodium borohydride, cannot be successful because other groups, readily reducible by [^{3}H]-sodium borohydride are already present in the membrane. Neither of the techniques was used in the subsequent study.

The Effects of dB-cAMP and Sq 20009 on the Agglutinability of the CHO Lines

The ability of concanavalin A and WGA to agglutinate the different CHO cell lines was tested on cells that had been grown in the presence and absence of 1mM 3':5'-cyclic AMP, 1mM dB-cAMP, 1 x 10^{-3} butyric acid and 10^{-4}M Sq 20009 (Table 8). In absence of any additives, all of the lines, including the elongated 24-2 were completely agglutinable by both lectins tested at concentrations of 25 and 125 µg/ml. The presence of dB-cAMP, however caused a marked loss in agglutinability of the H-7 and 24-2 lines to Con A, but had no measurable effect on K-1 and M-7. This was surprising since K-1 is the line most responsive to dB-cAMP in terms of morphological change; M-7 of course does not elongate under these conditions. Only H-7 showed a marked decreased agglutinability towards WGA and then only at the lowest concentration of lectin. 3':5'-cyclic AMP also caused a loss in concanavalin A-induced agglutinability of H-7, but had little influence on the other lines. Even in relation

TABLE 8. The effect of dB-cAMP, 3':5'-cyclic AMP butyric acid and Sq 20009 on the agglutination of the CHO cell lines by concanavalin A and WGA

	TREATMENT*	% AGGLUTINATION BY WGA (μg/ml)		CON A (μg/ml)	
		25	125	25	125
H-7	No additions	100	100	100	100
	1 mM dB-cAMP	0	90	0	0
	1 mM c-AMP	---	---	25	65
	1 mM Butyric acid	85	100	90	100
	0.1 mM Sq 20009				
M-7	No additions	100	100	100	100
	1 mM dB-cAMP	90	100	100	100
	1 mM cAMP	---	---	100	100
	0.1 mM Sq 20009	10	100	10	100
24-2	No additions	100	100	90	100
	1 mM dB-cAMP	100	100	30	60
	1 mM cAMP	---	---	75	100
	0.1 mM Sq 20009	15	100	20	20
K-1	No additions	90	100	100	100
	1 mM dB-cAMP	90	100	100	100
	1 mM cAMP	---	---	100	100
	0.1 mM Sq 20009	60	---	80	---

* Description of treatment of cells after they reached the desired density. Unless otherwise indicated the cells were grown without additions to the growth medium except those indicated in the section on cell maintenance.

to H-7, it was less potent than dB-cAMP. This may be due to the greater stability of the butyryl analog in the cell's growth medium. Sq 20009 induced some loss in both WGA and concanavalin A agglutinability of the 24-2 and M-7 lines but had no measurable influence on K-1 or H-7. Butyric acid, which was included as a control to test the effect of possible decomposition products of dB-cAMP, did not alter the agglutinability of CHO-H-7 and can be eliminated as a non-specific cause of changes observed in the presence of dB-cAMP.

The changes in agglutinability observed are not due to an increased number of binding sites upon treatment with the drugs, as is shown in Table 9. In fact, the 24-2 line has twice as many binding sites upon treatment with dB-cAMP, whereas it is less agglutinable. It is clear from the results that there is no satisfactory correlation between the morphological responsiveness of the different cells to various drugs and their ability to undergo drug-induced losses in agglutinability. We conclude, therefore, that the two phenomena are separable.

Effect of colchicine, low temperature and enzyme treatment on agglutinability of CHO cells

Colchicine is known to disrupt intracellular microtubules and has a direct effect on cellular processes in which microtubules are involved. CHO cells (H-7) were therefore grown in presence of 5×10^{-5}M colchicine for 5 h before being tested for agglutinability by low concentrations of concanavalin A and WGA. This treatment caused a marked loss in agglutinability when compared with controls (Table 10). The change was of about the same magnitude as that induced by dB-cAMP alone. If colchicine is added to cells growing in presence of dB-cAMP, the cells lose their elongated shape, suggesting that microtubules are

Table 9. The effect of dB-cAMP and Sq 20009 on the binding of [^{3}H]-concanavalin A by the CHO cell lines.

Cell Line	Drug Added to Medium	cpm/10^7 Cells*
H-7	No	67227
	10^{-3} M dB-cAMP	51915
K-1	No	61228
	10^{-3} M dB-cAMP	51513
K-1-M-7	No	79875
	10^{-4} M Sq 20009	53460
K-1-24-2	No	37443
	10^{-3} M dB-cAMP	81897
	10^{-4} M Sq 20009	46055

* Average of 5 determinations.

TABLE 10. The effect of temperature, enzyme treatment and colchicine on lectin-induced agglutination of CHO H-7 by concanavalin A and WGA.

	Treatment*	% Agglutination by 25 µg/ml Con A	WGA
a	Untreated cells	90	90
b	Cells incubated at 0° for 15 min before assay	10	10
c	Cells incubated at 0° for 15 min and then returned to 37° for 15 min before assay	90	85
d	Cells grown on 10^{-3} M dB-cAMP	20	10
e	Cells grown on 10^{-3} M dB-cAMP and treated with 10 µg/ml chemotrypsin for 5 min at 22°C before assay	100	100
f	Cells grown on 10^{-3} M dB-cAMP and treated with 5×10^{-5} M colchicine for 4 h before assay	20	0
g	Cells grown on normal medium and treated with 5×10^{-5} M colchicine 4 h before assay	30	20
h	Cells grown on normal medium and treated with 10 µg/ml neuraminidase for 15 min before assay	90	90

involved in the morphological response. The agglutinability of these cells is very low.

The influence of low temperature on the agglutinability of CHO cells was similar to that observed on other transformed mammalian cell lines (132). A reversible decrease in both concanavalin A and WGA-induced agglutinability was observed if the cells were cooled to 0°C for 15 min before testing (Table 10).

Brief exposure of the cells to low concentrations of chymotrypsin reversed the loss in agglutinability induced by dB-cAMP. Similar results have been observed on untransformed fibroblasts which are normally not agglutinable but which will respond to the lectins after brief trypsinization (132).

The presence of sialic acid on certain glycopeptides is known to enhance the binding of the natural ligand N-acetylgalactosamine to WGA (149). In addition, a decrease in sialic acid on certain glycopeptides has been observed after the H-7 line were exposed to 3':5'-cyclic AMP (46,47). However, removal of sialic acid groups from the surface of CHO-cells by means of neuraminidase did not mimic the effects of dB-cAMP and the cells remained completely agglutinable.

Time course for changes in agglutination and effects of inhibitors of protein and RNA synthesis

The agglutinability of cells was determined at fixed time intervals after dB-cAMP was added to or removed from the growth medium. From the control values in Table 11 it is clear that the response of the cells to the drug was extremely rapid. Within 4 h of adding dB-cAMP, line H-7 completely lost its agglutinability towards both concanavalin A and WGA when the lectins were tested at concentrations of 50 and 125 µg/ml. In the

TABLE 11. The temporal effects of addition and removal of dB-cAMP on the agglutination of CHO-H-7 by concanavalin A and WGA

TREATMENT	% AGGLUTINATION BY WGA (μg/ml)		CON A (μg/ml)	
	50	125	50	125
No additions	95	100	100	100
1 mM dB-cAMP was added:				
4 hrs before assay	10	10	10	10
2 hrs before assay	20	80	70	80
1 hr before assay	25	90	70	90
Cells were grown on 1 mM dB-cAMP for 48 hrs. The medium was changed to normal medium:				
4 hrs before assay	90	95	95	100
2 hrs before assay	80	95	100	100
1 hr before assay	50	90	75	100

reverse-type of experiment, when the cells were transferred to normal medium after being maintained on dB-cAMP, complete agglutinability was restored within 1 to 2 h.

To see if protein or RNA synthesis were required for the changes in agglutinability to occur we added a number of metabolic inhibitors to the cells at intervals before assaying for agglutination (Table 12). Cyclohexamide, actinomycin D and cordycepin all inhibited the loss in agglutinability normally caused by dB-cAMP if they were introduced to the cells simultaneously with the nucleotide. It seems likely that both RNA and protein synthesis are required in order for dB-cAMP to induce the non-agglutinable state.

Cells were also observed to become fully agglutinable after they had been grown continuously on dB-cAMP but cyclohexamide, actinomycin D or cordycepin added 4 h before assay. It would appear that even after the non-agglutinable state had been achieved, protein synthesis had to continue for the condition to be maintained. We conclude that transcriptive events are required not only to induce the non-agglutinable state but probably also to maintain it.

In the final set of experiments reported in Table 12 we tested the ability of inhibitors to counteract the return of the cells to the fully agglutinable state after they had been removed from medium containing dB-cAMP. This reversion is normally very rapid (see control values in Table 11) and it was not prevented by cyclohexamide, actinomycin D or cordycepin.

Similar experiments to the ones described above have been carried out to determine the effects of cycloheximide and actinomycin D on the loss of agglutinability induced by dB-cAMP or SQ 20009 on line 24-2.

TABLE 12. The effects of inhibitors of protein and RNA synthesis on dB-cAMP induced loss of agglutination of CHO-H-7 by concanavalin A and WGA

Treatment	Control (no inhibitor added)		Cyclohexamide (2 µg/ml)		Actinomycin D (0.5 µg/ml)		Cordycepin (2 µg/ml)	
	(% Agglutination by Lectin)							
	Con A	WGA	Con A	WGA	Con A	WGA	Con A	WGA
Inhibitor added:								
a) 4 h before assay	95	95	95	90	100	100	90	85
Inhibitor added simultaneously with dB-cAMP:								
a) 1 h before assay	70	90	90	95	100	100	90	80
b) 2 h before assay	70	80	90	100	95	90	--	--
c) 4 h before assay	10	10	90	90	100	100	95	90
Cells grown continuously on dB-cAMP for 48 h and inhibitor added:								
a) 4 h before assay	10	5	90	90	100	100	80	80
Cells grown continuously on dB-cAMP for 48 h and then changed to normal medium plus inhibitor:								
a) 1 h before assay	75	50	50	10	90	90	50	50
b) 2 h before assay	100	80	90	90	95	95	--	--
c) 4 h before assay	95	90	90	90	95	90	90	85

The results are summarized in Tables 13 and 14. Again, the cells lost their agglutinability within 4 h of being exposed to the drug and regained it within one hour after its removal from the medium. Cyclohexamide and actinomycin D inhibited the first process (i.e. the loss in agglutinability) but had no effect on the second so that agglutinability was regained normally.

The effect of dB-cAMP and Sq 20009 on the fucose containing glycopeptides of the plasma membrane

The effect of dB-cAMP has been investigated most intensively on line H-7. Cell surface glycopeptides from cells under contrasting conditions were compared by means of double-label experiments using either [^{14}C] or [^{3}H]-L-fucose. This sugar is incorporated specifically into fucosyl units of the complex carbohydrates of CHO cells, just as it is in other higher organisms. Figure 31 shows a paper-chromatographic separation of the acid hydrolysate of the pronase-digested trypsinates of the H-7 cell surface. It can be seen that upon extensive hydrolysis most of the label is found in peak B and an earlier peak A is probably due to partial hydrolysis products since it disappears upon extensive hydrolysis. Peak B corresponds to fucose and peak C is probably due to acid hydrolysis of fucose which gives a furanose.

Figure 32 shows a typical radioactivity profile obtained when the pronase-digested trypsinates of the cell surface of H-7 cells grown in presence or absence of dB-cAMP are co-chromatographed on a column of Sephadex G-50. The normal cells invariably contribute more radioactivity to the leading edge of the main radioactive peak, indicating they are of higher molecular weight. If the fractions in this main peak are pooled, dialyzed and subjected to ion-exchange chromatography

TABLE 13. The effects of cyclohexamide and actinomycin D on Sq 20009 induced loss of agglutionation of CHO-K-1-24-2 by concanavalin A and WGA

Treatment	Control (no inhibitor added)		Cyclohexamide (2 µg/ml)		Actinomycin D (0.5 µg/ml)	
	(% Agglutionation by Lectin)					
	Con A	WGA	Con A	WGA	Con A	WGA
Inhibitor was added 4 h before assay	90	85	90	80	90	90
Inhibitor added simultaneously with 0.1 mM Sq 20009 4 h before assay	90	85	90	80	90	90
Cells grown continuously on 0.1 mM Sq 20009 for 48 h and inhibitor added 4 h before assay	5	25	90	90	30	15
Cells grown continuously on Sq 20009 for 48 h and then changed to normal medium plus inhibitor 4 h before assay	90	80	90	95	80	80

TABLE 14. The effects of cyclohexamide and actinomycin D on dB-cAMP induced loss of agglutination of CHO-24-2 by Con A and WGA

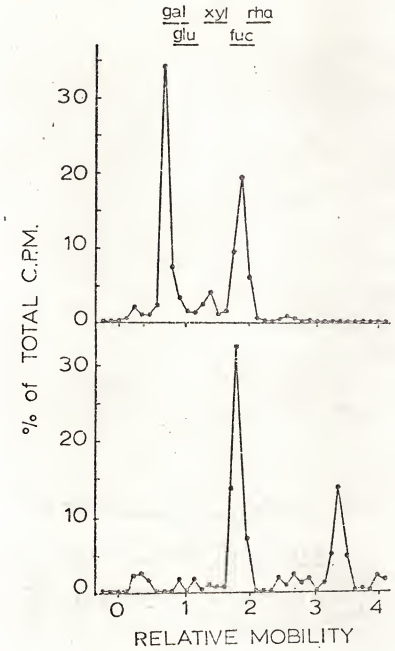
	Control (no inhibitor added)		Cyclohexamide (2 µg/ml)		Actinomycin D (0.5 µg/ml)	
	(% Agglutination by Lectin)					
Treatment	Con A	WGA	Con A	WGA	Con A	WGA
Inhibitor was added 4 h before assay	90	85	90	80	90	80
Inhibitor added simultaneously with 1 mM dB-cAMP:						
a) 1 h before assay	--	--	80	75	90	80
b) 4 h before assay	90	85	90	80	90	75
Cells grown continuously on 1 mM dB-cAMP for 48 h and inhibitor added:						
a) 4 h before assay	25	10	90	80	70	30
Cells grown continuously on 1 mM dB-cAMP for 48 h and then changed to normal medium plus inhibitor:						
a) 1 h before assay	--	--	90	80	50	30
b) 4 h before assay	90	80	90	80	90	80

Figure 31. Paper chromatographic separation of hydrochloric acid hydrolysate of fucose labeled glycopeptides.

Glycopeptides labeled with [^{3}H]-fucose, released from the cell surface by trypsin, were pronase digested, desalted and chromatographed on Sephadex G-50. The eluate of this column, except the included volume was hydrolyzed as described below. The hydrolyzate was spotted on Whatman No. 1 chromatography paper. Standards were chromatographed at the same time, using a solvent system of butanol:acetic acid:water/37:25:9. The strip containing the hydrolyzate was cut into 1 cm fractions and counted for radioactivity. The standards were visualized with a silver nitrate/alkaline spray.

Top - Hydrolysis in 0.1 N hydrochloric acid for 24 h at 100°C in presence of 1 mg Domex H^{+}.

Bottom - Hydrolysis in 0.5 N hydrochloric acid for 48 h at 100°C in presence of 1 mg Domex H^{+}.


gal xyl rha
glu fuc
30
20
10
0
% of TOTAL C.P.M.
30
20
10
0
0 1 2 3 4
RELATIVE MOBILITY

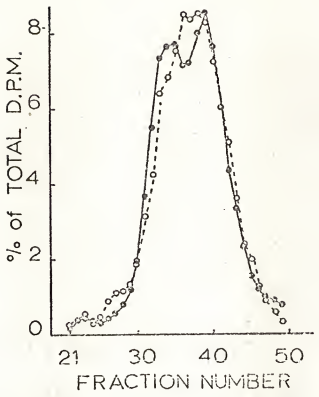


Figure 32. G-50 Sephadex elution profile of pronase-digested mixed trypsinates of CHO-H-7.

The cells were grown in the presence of [^{14}C]-fucose (●) or [^{3}H]-fucose and 1 mM dB-cAMP. When the cultures reached about 90% confluency the cells were trypsinized. The trypsinates were pronase digested, desalted and chromatographed on a Sephadex G-50 column.

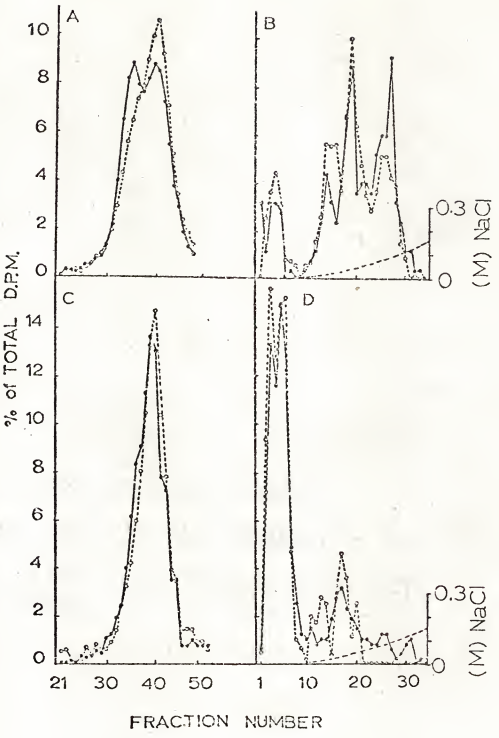
Figure 33. The effect of hydrochloric acid hydrolysis on fucose-containing cell surface glycopeptides.

a H-7 cells were grown for 48 h on medium containing [^{14}C]-fucose (●) and 1 mM dB-cAMP. 4 h before trypsinization this medium was replaced by medium with [^{3}H]-fucose (○).

b The fractions 20-50 of a were pooled, concentrated under vacuum, desalted and a portion was chromatographed on a DEAE-cellulose ion exchange column.

c The fractions 20-50 of a were pooled and an aliquot was incubated with 0.1 N HCl at 80°C for 1 h and rechromatographed on the same G-50 Sephadex column.

d The fractions 20-50 of c were pooled and chromatographed on a DEAE-cellulose ion exchange column.


A
B
C
D
10
8
6
4
2
0
14
12
% of TOTAL D.P.M.
0.3
(M) NaCl
21
30
40
50
1
10
20
30
FRACTION NUMBER

on DEAE-cellulose, a complex elution pattern similar to that shown in Figure 33b is observed. Although some radioactivity does not bind to the column and it appears in the initial buffer wash, a large proportion is acidic in nature and eluted during the salt gradient. The normal cells always contribute most extensively to the later eluting and presumably most acidic fractions, while the treated cells give rise to a greater proportion of radioactivity in the early-eluting peaks. Treatment of the glycopeptides with 0.1 N HCl at 80° for 30 min (Figure 33c,d) or with neuraminidase (Figure 34) in order to remove sialic acid groups, produces glycopeptides of uniformly lower molecular weight which were indistinguishable by gel-permeation. Similarly, if the cells are grown in presence of low concentrations of neuraminidase, the glycopeptides from normal cells are of considerably lower molecular weight and charge as determined by Sephadex G-50 (Figure 34). Therefore, it seems likely that cells grown in presence or absence of dB-cAMP differ in the amount of sialic acid that is bound to their fucose-containing glycopeptides.

Effect on all CHO lines

Figure 35 is a composite from an extensive experiment in which the fucose-containing glycopeptides were compared for all of the cell lines grown in presence or absence of dB-cAMP or Sq 20009. Additional unpublished experiments by Dr. R. M. Roberts and Alonzo Walker have also been carried out to test the effects of the natural nucleotide 3':5'-cyclic AMP. Further, it has been shown that all of the lines grown under normal conditions have a fairly similar spectrum of fucose containing glycopeptides at their surface (R. M. Roberts, unpublished results). For example, co-chromatography of glycopeptides from the elongated line

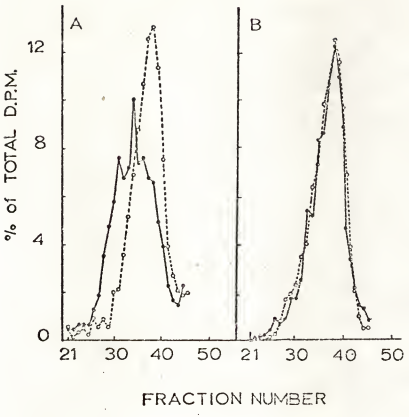


Figure 34. The effect of neuraminidase on fucose-containing cell surface glycopeptides.

a Cells were grown in presence of [^{3}H]-fucose (0) or [^{14}C]-fucose (●) for 48 h. The ^{3}H labeled cells were incubated for 45 min with 2 units neuraminidase in PBS,pH 7.2, before trypsinization. The pronase digested trypsinates of both samples were mixed and co-chromatographed on a G-50 Sephadex column.

b The fractions 20-50 of the eluate from Figure 31a were pooled, desalted on a Bio-gel P-2 column and an aliquot was incubated with 0.2 units neuraminidase in 0.01 M sodium acetate buffer, pH 5.4, for 20 h. This sample was re-chromatographed on a G-50 Sephadex column.

Figure 35. Fucose-containing glycopeptides released from the surface of CHO cells by trypsinization.

The figures in column A indicate the effect of 1 mM dB-cAMP on the different cell lines, those in column B show the effect of 0.1 mM Sq 20009

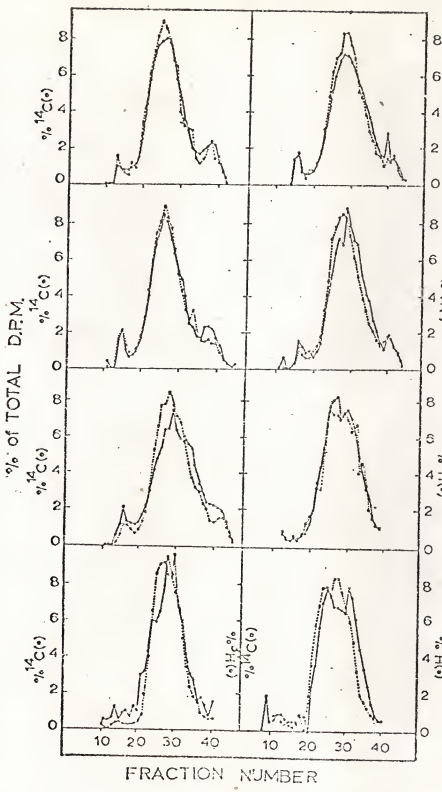
1) CHO-K-1 (●) ^{14}C = either dB-cAMP or Sq 20009, (○) ^{3}H = no addition.

2) CHO-K-1-M-7 (●) ^{14}C = either dB-cAMP or Sq 20009, (○) ^{3}H = no addition.

3) CHO-H-7 (●) ^{14}C = no addition, (○) ^{3}H either dB-cAMP or Sq 20009

4) CHO-K-1-24-2 (●) ^{14}C = either dB-cAMP or Sq 20009, (○) ^{3}H = no addition

See text for further explanation.


% of TOTAL D.P.M.
% 14C(•)
(•)H3 %
FRACTION NUMBER
10 20 30 40
0 2 4 6 8

24-2 with H-7, K-1 or M-7 gave mixed label profiles which coincided very closely.

Figure 35 indicates that only when H-7 and 24-2 are treated with dB-cAMP is there a shift in the glycopeptide pattern towards an average lower molecular weight (Figures 35-3a, 35-4a). Although the chromatographic resolution obtained here is not as good as that obtained in some of our later experiments, e.g. Figure 32, the leading edge of high molecular weight materials which were contributed by the untreated cells can be clearly discerned. It is of interest that K-1 which responds morphologically to dB-cAMP does not show any detectable shift in its fucose-containing glycopeptides. M-7 which is morphologically unresponsive to dB-cAMP but which elongates in presence of Sq 20009, also shows the changed glycopeptide composition only in presence of the diesterase inhibitor.

It became clear to us upon examination of the results that there was no clear-cut correlation between the morphological responsiveness of the cells to dB-cAMP, to Sq 20009 or to 3':5'-cyclic AMP itself, see Table 15. However, also included in the table is a summary of concanavalin A agglutination experiments reported earlier. Clearly a loss in lectin-induced agglutinability is invariably accompanied by a change in surface glycopeptide composition. For example, consider line H-7. This responds morphologically to dB-cAMP but only poorly if at all to Sq 20009 and 3':5'-cyclic AMP. On the other hand, it loses its concanavalin A-induced agglutinability in presence of dB-cAMP and 3':5'-cyclic AMP and only under the latter conditions is there a shift in the pattern of fucose-containing glycopeptides. This corelation, which we believe reflects a coordinate change in the surface of the cells, is discussed further in a later part of this dissertation.

TABLE 15. A Summary of the effect of 3':5'-cyclic AMP, dB-cAMP and Sq 20009 on the surface glycopeptides, agglutination and morphology of the CHO cell lines studied.

Effect of:	CHO Line K-1	H-7	M-7	24-2
3':5'-cyclic AMP addition to the medium on the:				
- surface glycopeptides	-	+	-	+
- Con A agglutination	-	+	-	+
- morphology	±	-	-	-
dB-cAMP addition to the medium on the:				
- surface glycopeptides	-	+	-	+
- Con A agglutination	-	+	-	+
- morphology	+	+	-	±
Sq 20009 addition to the medium on the:				
- surface glycopeptides	-	-	+	+
- Con A agglutination	-	-	+	+
- morphology	-	-	+	±

\+ indicates that: 1) there is a loss of the leading shoulder in the Sephadex G-50 elution pattern

2) the cell is not agglutinated by concanavalin A

3) the cell becomes more elongated

\- indicates no change

± change could not be established unequivocally.

Changes in the size of existing fucose-containing glycopeptides when either dB-cAMP or Sq 20009 are added to or removed from the growth medium

In this series of experiments we attempted to find out whether the change in size of the glycopeptides induced by altered growth conditions occurred on molecules already present in the membranes. In other words, could sialic acid be added to or be removed from existing glycopeptides when the cells were transferred to or from medium containing dB-cAMP or Sq 20009.

In the first experiment ^{3}H-labeled glycopeptides were obtained from lines H-7, M-7 and 24-2 maintained under normal growth conditions for 48 h in presence of L-[^{3}H]-fucose. Similar cells were maintained for 48 h in presence of L-[^{14}C]-fucose. However, 4 h before harvest, the radioactivity was removed and new medium containing either 1 mM dB-cAMP or 0.1 mM Sq 20009 was added. The trypsinates from the contrastingly labeled groups of cells were then mixed, treated with pronase and the glycopeptides co-chromatographed. The design of the experiment is illustrated diagramatically below.

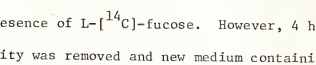
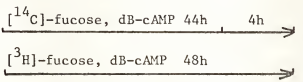


Figure 36 shows the radioactive patterns obtained. In each case there was very close coincidence between the ^{3}H and ^{14}C profiles. We conclude that the reductions in molecular weight of the glycopeptides that we have noted in the previous sections to be induced by dB-cAMP on 24-2 and H-7 and by Sq 20009 on M-7 and 24-2 are not due to removal of sialic acid groups from existing glycopeptides by an endogenous neuraminidase. If this had been the case we would have anticipated a shift in the ^{14}C-profile towards lower molecular weight.

In a second type experiment, using the dB-cAMP responsive line H-7, cells were grown normally for 24 h on [^{14}C]-L-fucose (10 μCi) and 1 mM dB-cAMP, then transferred to normal medium for either 4 or 15 h. Trypsinates were harvested at each of these times. As an internal standard used for comparison on the G-50 column, I included [^{3}H]-glycopeptides from cells grown continuously on dB-cAMP. The design of this experiment is illustrated below.



When the mixed label glycopeptides were chromatographed together on the G-50 column the pattern was typically that which distinguishes normal from dB-cAMP-treated cells, e.g. similar to Figure 32. Therefore, in this experiment, the average molecular weight of the [^{14}C]-labeled materials had increased during the 4 h following transfer to normal medium. Clearly some peak B lower molecular weight material laid down during the initial period on dB-cAMP was evidently converted to peak A higher molecular weight material following transfer.

These results are consistent with the theory that sialic acid residues can be added to existing glycoproteins already located at, or in transit to, the cell surface. In order to rule out the possibility that the modifications did not occur intracellularly on [^{14}C]-labeled glycoproteins being synthesized at the end of the labeling period on [^{14}C]-L-fucose, new medium lacking radioactivity but containing dB-cAMP was added for a 4 h interim period, before the addition of the [^{3}H]-L-fucose and normal medium. The results of this experiment are shown in Figure 37.

This figure clearly indicates that some glycopeptides can be modified after reaching the cell surface. However, there is not a complete

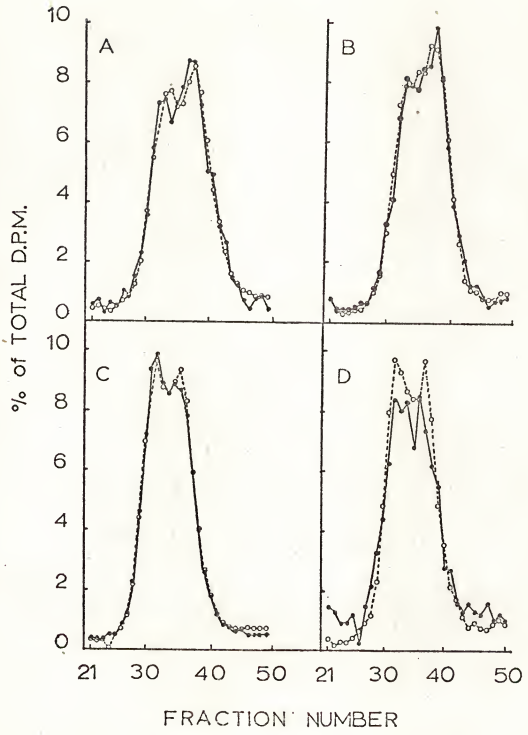
Figure 36. Fucose-containing glycopeptides released from the surface of CHO cells by trypsinization after different labeling conditions.

Cells were provided 5 μCi [^{14}C]-fucose (●) in the growth medium for 48 h. Four h before trypsinization the label was removed and a drug was added. Other cells were grown in the presence of 50 μCi [^{3}H]-fucose for 48 h. The cells were trypsinized and co-chromatographed on a Sephadex G-50 column.

a) H-7 cells, pre-labeled with [^{14}C]-fucose were provided with 1 mM dB-cAMP when the label was removed.

b) M-7 cells, pre-labeled with [^{14}C]-fucose were provided with 0.1 mM Sq 20009 when the label was removed.

c-d) 24-2 cells, pre-labeled with [^{14}C]-fucose were provided with 1 mM dB-cAMP (c), or 0.1 mM Sq 20009 (d) when the label was removed.


A
B
C
D
10
8
6
4
2
0
% of TOTAL D.P.M.
21
30
40
50
FRACTION NUMBER

shift in the pattern to that of cells grown continuously in the absence of dB-cAMP. It therefore seems probable that some sialic acid is added before the glycopeptides reach the cell surface.

The effect of colchicine on the size of L-fucose containing surface glycopeptides

As discussed earlier, colchicine disrupts microtubules in the cell by binding to the dissociated subunits. Microtubules have been implicated in the reduction of the mobility of surface glycopeptides (67). I therefore wanted to study the effect of this drug on the addition of sialic acid to existing glycopeptides at the cell surface.

Cells were grown in the presence of radiolabeled fucose and dB-cAMP for 24 h. Colchicine (5×10^{-5}M) was added for 4 h before trypsinization. Such a sample was co-chromatographed with trypsin digest of cells of contrasting labeled with fucose in the presence, Figure 38a, or absence of dB-cAMP, Figure 38b. Clearly, upon growth in the presence of colchicine the average molecular weight of fucose containing glycopeptides is higher than that of cells grown exclusively in the presence of dB-cAMP, but lower than that of cells grown on medium which did not contain dB-cAMP. These results are again consistent with the hypothesis that increased mobility of surface glycoproteins, in these experiments induced by colchicine, allow further glycosylation of surface glycopeptides.

The effects of cyclohexamide on the size of the L-fucose containing glycopeptides

In order to study further whether sialic acid could be added to existing glycopeptides already present in the membranes of CHO cells, the following experiment was designed. Cells were grown in presence of [^{14}C]-fucose and 1 mM dB-cAMP for 48 h and then transferred to medium

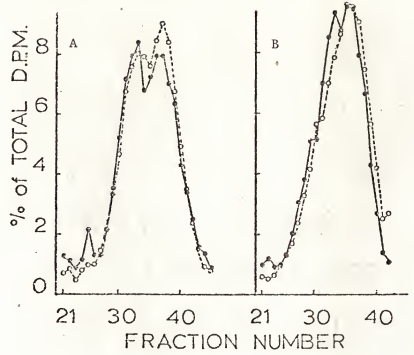


Figure 37. Fucose-containing glycopeptides released from the surface of CHO-H-7 after removal of dB-cAMP from the growth medium.

Cells were grown in the presence of 1 mM dB-cAMP and 5 μCi [^{14}C]-fucose (O) for 24 h. Six hours before trypsinization of the cells new medium containing 1 mM dB-cAMP was added for 2 (A) and 4 (B) h. Subsequently the cells were incubated for 2 h with 50 μCi [^{3}H]-fucose (O) in the absence of dB-cAMP. The cells were trypsinized and the trypsinates were co-chromatographed on Sephadex G-50.

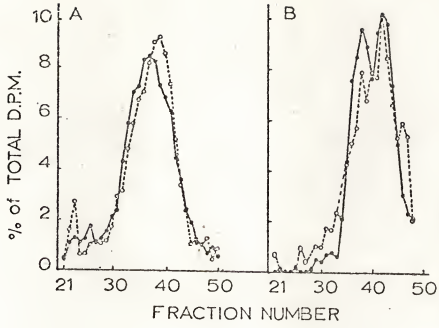


Figure 38. The effect of colchicine on the fucose-containing surface glycopeptides of CHO-H-7.

A) Cells were grown in the presence of 1 mM dB-cAMP and 5 μCi [^{14}C]-fucose (●) or 20 μCi [^{3}H]-fucose (○). Four h before trypsinization the ^{14}C-labeled cells were given fresh medium containing 1 mM dB-cAMP and 5 x 10^{-5} M colchicine. The pronase-digested trypsinates of these samples were co-chromatographed on a Sephadex G-50 column.

B) Cells were grown in the presence of 1 mM dB-cAMP and 20 μCi [^{3}H]-fucose (○) or in the presence of 5 μCi [^{14}C]-fucose (●), without dB-cAMP for 48 h. Four h before trypsinization the ^{3}H-labeled cells were given fresh medium containing 1 mM dB-cAMP and 5 x 10^{-5} M colchicine. The pronase-digested trypsinates of these samples were co-chromatographed on a Sephadex G-50 column.

containing cyclohexamide for 4 h. The trypsinates were then harvested, mixed with ^{3}H-material from cells maintained normally on dB-cAMP and chromatographed on Sephadex G-50 (Figure 39). Clearly in the absence of continued protein synthesis, there was a relatively major shift in the glycopeptide pattern towards components of higher molecular weight.

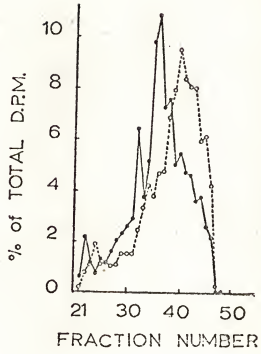


Figure 39. The effect of cyclohexamide on the fucose-containing surface glycopeptides of CHO-H-7.

Cells were grown for 48 h on medium containing 5 μCi [^{14}C]-fucose (●) and 1 mM dB-cAMP. Four h before trypsinization these cells were given fresh medium containing 1 mM dB-cAMP and 2 μg/ml cyclohexamide. This trypsinate was mixed with that of cells grown continuously in the presence of 10 μCi [^{3}H]-fucose and 1 mM dB-cAMP, and co-chromatographed on a Sephadex G-50 column.

DISCUSSION

There has been a considerable interest in the relationship of 3':5'-cyclic AMP and the control of metabolic processes. This compound acts as a "second messenger" in the action of numerous hormones and has been proposed to be the central agent integrating the control of many distinct, unrelated intracellular processes. This was termed "pleotypic control" (4). The observation that 3':5'-cyclic AMP is present in lower amounts in transformed cells than in their normal counterparts (30) and the further observation that addition of exogenous 3':5'-cyclic AMP could reverse the transformed phenotype (39,40) suggested that this compound may be involved in the process of transformation.

In our studies I have preferred to test the effects of an analog of 3':5'-cyclic AMP, dB-cAMP, which is more stable than the natural nucleotide, and Sq 20009, an inhibitor of 3':5'-cyclic AMP phosphodiesterase activity. The latter compound probably increases the intracellular concentration of 3':5'-cyclic AMP due to these inhibitory properties. In this study I have examined the responses of four clones of CHO cells to these drugs in an attempt to evaluate whether there were changes which could be considered to be equivalent to what Hsie and Puck (2) described as "reverse transformation."

Growth of the cells was unaffected by the presence of dB-cAMP in the medium. They reached the same final cell density and appeared to

have the same cell population doubling time. Moreover most or all of the cells appear to pass through the cell cycle and growth of the population is not due to only a few cells with extremely short doubling times. This was a useful property since we were then able to compare cells in similar growth state. Others have indicated that dB-cAMP slows or even prevents proliferation of certain mammalian cell lines when provided at concentrations as high as these used in our studies (32-34). It has also been suggested that it is the level of intracellular 3':5'-cyclic AMP which determines density-dependent inhibition of cell division (34). However, it should be emphasized that some of the earlier work employed the incorporation of ^{3}H-thymidine to determine relative rates of cell division between groups of cells. Since dB-cAMP affects the thymidine uptake differently in different cell lines, such a technique clearly cannot be used to measure relative rates of growth. By contrast, our results suggest that increased intracellular 3':5'-cyclic AMP levels do not reduce the growth rate of CHO cells nor induce a condition analogous to contact inhibition of growth. Our experiments indicated that although cell surface changes could be demonstrated using the highly sensitive agglutination techniques, dB-cAMP did not induce any major differences in the pattern of plasma membrane proteins detected using the double-labeling technique with L-leucine. Neither was there any evidence for a change in surface architecture revealed by the pattern of "exposed" proteins labeled by iodine in presence of H_2O_2 and peroxidase. The nucleotide did not induce the formation of a high molecular weight (>200,000), iodinatable, trypsin-sensitive protein which has been considered to be almost invariable characteristic of non-transformed fibroblasts, but which is absent in transformed cells (120). Again this

suggests that dB-cAMP is not eliminating all features of the transformed phenotype in these CHO cells.

Pastan and his collaborators have recently suggested that high levels of intracellular 3':5'-cyclic AMP will render a cell minimally agglutinable with Con A (150). However, it will be noted that high levels of dB-cAMP may be added to two of the lines investigated in this work, K-1 and M-7, without modifying the agglutinability of the cell line with concanavalin A. Of special interest in this case is the line K-1 which elongates dramatically but does not lose its agglutinability upon addition of dB-cAMP to its growth medium. Thus these lines also demonstrate that there is no necessary correlation between the morphology of a cell line and its relative agglutinability with concanavalin A or WGA. This conclusion is also reinforced by line 24-2 which is permanently elongated yet fully agglutinable. Similarly M-7 which does not respond morphologically to 3':5'-cyclic AMP does lose its agglutinability in its presence. Further, others have shown that the morphological changes induced in CHO cells (48,50,51) do not require nuclear events, whereas we have demonstrated that the loss of agglutinability requires both protein and RNA synthesis. Clearly these two features normally characteristic of the transformed phenotype, a compact epithelial-like shape and sensitivity to agglutination by plant lectins, are separable phenomena.

Modification in agglutinability of these CHO cell lines by concanavalin A following the addition or removal of dB-cAMP to the growth medium was: (1) not dependent on a change in the number of lectin receptors, (2) not dependent on a modification of the fatty acid composition of the plasma membrane, and (3) not dependent on a change in the total amount of surface sialic acid, since normal cells remained fully agglutinable

following treatment with neuraminidase. However, a correlation does exist between loss in agglutinability and loss of sialic acid from certain cell surface glycoproteins, as will be discussed later.

Maintenance of the non-agglutinable state by cells grown in the presence of dB-cAMP, or the ability of cells to attain this state following addition of dB-cAMP was: (1) dependent on protein and RNA synthesis and (2) dependent on a trypsin-labile and presumably surface membrane protein. On the other hand, the change from the non-agglutinable state to the agglutinable state following removal of dB-cAMP or Sq 20009 was not dependent on protein or RNA synthesis.

Since the change to the non-agglutinable state and its reversal happen quickly, it must be assumed that very rapid changes, presumably in the mobility of the lectin receptors occur when cells are confronted with altered levels of 3':5'-cyclic AMP. Moreover, it has to be assumed that the components that are responsible for maintaining the non-agglutinable state turn over very rapidly.

As stated earlier, however, we have not been able to demonstrate major differences in the pattern of plasma membrane proteins between the agglutinable and non-agglutinable states or between the lines which differ in their responsiveness to dB-cAMP. Neither have we been able to detect a protein which turns over more rapidly than other components in the membrane of treated cells (J. van Veen, R. M. Roberts and K. D. Noonan, unpublished results). Therefore, it has not been possible to decide what precise components are involved in maintaining the non-agglutinable state in these treated CHO cells. It must be assumed that a new structural protein, possibly a very minor one, or an induced enzyme is involved. There is some evidence that a cellular micro-

architecture comprised of microtubules or microfilaments (or possibly both) has a role in restricting the lateral movement of proteins in the plasma membrane since drugs which disrupt these structures also affect such processes as agglutinability of transformed cells and capping in lymphocytes (92-98). It is possible that the new protein is a "linkage" protein between the membrane polypeptides and such restraining structures in the cytoplasm. Alternatively, the induction of the non-agglutinable state may require such events as phosphorylation or other modifications of existing proteins, processes which in turn may require the induction of specific enzymes with short half-lives.

One surface modification which seems to occur concomitantly with the change in agglutinability is the decrease in sialic acid associated with the fucose-containing glycopeptides that can be released from the cell surface by incubation with trypsin. It seems likely that this association of two modifications in surface characteristics is not a chance event. On the other hand, it is most unlikely that these sialic acid-containing glycopeptides are the lectin receptors for two reasons: (1) cells treated with neuraminidase and which have lost their sialic acid from these glycopeptides remain fully agglutinable by concanavalin A, (2) the sialic acid glycopeptides themselves do not bind efficiently to concanavalin A immobilized on Sepharose (R. M. Roberts, J. Van Veen, unpublished results). Similar differences in fucose-containing glycopeptides have been shown to distinguish transformed fibroblasts from their normal counterparts (9-12). I believe that these results are also consistent with the hypothesis that the protein components of the dB-cAMP treated cells are less mobile, i.e. less able to migrate laterally than those of controls. I propose that in the untreated cell, incomplete

glycopeptides and appropriate glycosyl transferases are able to make ready contact so that terminal sialic acid groups are added to complete the carbohydrate chain. If protein movement is restricted, however, this event may occur less readily so that a population of incomplete macromolecules results.

Glycosyl transferases capable of utilizing exogenous sugar nucleotides including CMP-N-acetyl neuraminic acid are present at the surface of cultured mammalian cells. Their presence there may be the result of movement of intracellular membrane to the surface via an assembly line process in which case their location may have no functional significance. On the other hand, others have proposed that they continue to function (122-124). However, it is generally assumed that highly charged, labile molecules such as sugar nucleotides are unlikely to persist outside the environment of the cell. Nevertheless, it cannot be ruled out that the glycosyl donor in these reactions is not a glycolipid or that reaction does not involve transglycosylation from other glycoproteins.

Our results certainly indicate that sialic acid can be added to existing surface glycopeptides in the absence of dB-cAMP but not in its presence. The results with colchicine are also consistent with the idea that the degree of mobility of membrane proteins affects glycosylation reactions. This drug disrupts microtubules and causes a single cap-like patch of lectin, and presumably other proteins, receptors to form in treated cells (96). Presumably all of the restraints on mobility are relieved. As expected completion of the glycopeptides occurs under these circumstances even if dB-cAMP is present. On the other hand, agglutination is inhibited presumably because the opportunity for multiple cross-links to form between cells is reduced.

In conclusion, therefore, we believe that in these experiments dB-cAMP induces the formation of some unstable component(s) which has a role in controlling the lateral mobility of lectin receptors and probably other proteins in the plasma membrane of susceptible cells. We believe that the altered composition of fucose-containing glycopeptides and changed susceptibility of the cells to lectin-induced agglutination are related phenomena and a direct result of these 3':5'-cyclic AMP mediated events. Moreover, since these changes also occur following cell transformation, identical processes may be involved.

BIBLIOGRAPHY

1. Kao, F. T. and Puck, T. T. (1968) Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. 60:1275-1281.

2. Hsie, A. W. and Puck, T. T. (1971) Morphological transformation of Chinese hamster cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone. Proc. Natl. Acad. Sci. 68:358-361.

3. Hsie, A. W., Jones, C. and Puck, T. T. (1971) Further changes in differentiation state accompanying the conversion of Chinese hamster cells to fibroblastic form by dibutyryl adenosine cyclic 3':5'-monophosphate and hormones. Proc. Natl. Acad. Sci. 68:1648-1652.

4. Hershko, A., Mamont, P., Shield, R. and Tomkins, G. M. (1971) Pleiotypic response. Nature New Biol. 232:206-211.

5. Bader, J. P. and Brown, N. R. (1971) Induction of mutations in an RNA tumor virus by an analog of a DNA precursor. Nature New Biol. 234:11-12.

6. Aaron, S. A. and Todaro, G. J. (1968) Basis for the acquisition of malignant potential by mouse cells cultivated in vitro. Science 162:1024-1026.

7. Black, P. H. (1968) The oncogenic DNA viruses: a review of in vitro transformation studies. Ann. Rev. Microbiol. 22:391-426.

8. Zarling, J. M. and Tevethia, S. S. (1971) Expression of concanavalin A binding sites in rabbit kidney cells infected with vaccinia virus. Virology 45:313-316.

9. Buck, C. A., Glick, M. C. and Warren, L. (1970) Comparative study of glycoproteins from the surface of control and R S virus transformed hamster cells. Biochem. 9:4567-4576.

10. Warren, L., Fuhrer, J. P. and Buck, C. A. (1972) Surface glycoproteins of normal and transformed cells: a difference determined by sialic acid and a growth-dependent sialyl transferase. Proc. Natl. Acad. Sci. 69:1838-1842.

11. Warren, L., Fuhrer, J. P. and Buck, C. A. (1973) Surface glycoproteins of cells before and after transformation by oncogenic viruses. Fed. Proc. 32:80-85.

12. Buck, C. A., Fuhrer, J. P., Soslau, G. and Warren, L. (1974) Membrane glycopeptides from subcellular fractions of control and virus-transformed cells. J. Biol. Chem. 249:1541-1550.

13. Pasternak, T., Sutherland, E. W. and Henion, W. F. (1962) Derivatives of cyclic 3':5'-adenosine monophosphate. Biochim. Biophys. Acta 65:558-560.

14. Robinson, G. A., Butcher, R. W. and Sutherland, E. W. (1968) Cyclic AMP. Ann. Rev. Biochem. 37:149-174.

15. Menaham, L. A., Hepp, K. D. and Wiesland, O. (1969) Liver 3':5'-nucleotide phosphodiesterase and its activity in rat liver perfused with insulin. Eur. J. Biochem. 8:435-443.

16. Heersche, J. N. M., Fedak, S. A. and Aurback, G. D. (1971) The mode of action of dibutyryl adenosine 3':5'-monophosphate on bone tissue in vitro. J. Biol. Chem. 246:6770-6775.

17. Kaukel, E. and Hilz, H. (1972) Permeation of dibutyryl cAMP into HeLa cells and its conversion to monobutyryl cAMP. Biochem. Biophys. Res. Commun. 46:1011-1018.

18. Kaukel, E., Fuhrmann, U. and Hilz, H. (1972) Divergent action of cAMP and dibutyryl cAMP on macromolecular synthesis in HeLa S3 cultures. Biochem. Biophys. Res. Commun. 48:1516-1524.

19. d'Armiento, M., Johnson, G. S. and Pastan, I. (1972) Regulation of adenosine 3':5'-cyclic monophosphate phosphodiesterase activity in fibroblasts by intracellular concentrations of cyclic adenosine monophosphate. Proc. Natl. Acad. Sci. 69:459-462.

20. Hsie, A. W., Kawashima, K., O'Neill, P. J. and Schroder, C. H. (1975) Possible role of adenosine cyclic 3':5' monophosphate phosphodiesterase in the morphological transformation of CHO cells mediated by N^6,O^2-dibutyryl adenosine cyclic 3':5'-monophosphate. J. Biol. Chem. 250:984-989.

21. Ryan, W. L. and Kurick, M. A. (1972) Adenosine 3':5'-monophosphate and N^6-2'-O-dibutyryl adenosine 3':5'-monophosphate transport in cells. Science 177:1002-1003.

22. Papahadjopoulos, D., Poste, G. and Mayhew, E. (1974) Cellular uptake of cyclic AMP captured within phospholipid vesicles and effect on cell growth behavior. Biochim. Biophys. Acta 363:404-418.

23. De Asua, L., Surian, S., Flawia, M. and Torres, N. (1973) Effect of insulin on the growth pattern and adenyl cyclase activity of BHK fibroblasts. Proc. Natl. Acad. Sci. 70:1388-1392.

24. Reddi, P. K. and Constantinides, S. M. (1972) Partial suppression of tumor production by dibutyryl cyclic AMP and theophylline. Nature 238:286-287.

25. Burger, M. M., Bombik, B. M., Breckenridge, B. M. and Sheppard, J. R. (1972) Growth control and cyclic alterations of cAMP in the cell cycle. Nature New Biol. 239:161-165.

26. Lieberman, I. (1959) Growth factors for mammalian cells in culture. J. Biol. Chem. 234:2754-2758.

27. Illiano, G. and Cuatrecasas, P. (1972) Modulation of adenylate cyclase activities in liver and fat cell membranes by insulin. Science 175:906-908.

28. O'Neill, P. J., Shchroder, C. H. and Hsie, A. W. (1975) Hydrolysis of butyryl derivatives of adenosine cyclic 3':5' monophosphate by CHO cell extract and characterization of the products. J. Biol. Chem. 250:990-995.

29. Seifert, W. and Paul, D. (1972) Levels of cyclic AMP in sparse and dense cultures of growing and quiescent 3T3 cells. Nature New Biol. 240:281-283.

30. Otten, J., Johnson, G. S. and Pastan, I. (1971) Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. Biochem. Biophys. Res. Commun. 44:1192-1198.

31. Otten, J., Johnson, G. S. and Pastan, I. (1972) Regulation of cell growth by cyclic adenosine 3':5'-monophosphate. J. Biol. Chem. 247:7082-7087.

32. Johnson, G. S. and Pastan, I. (1972) Role of 3':5'-adenosine monophosphate in regulation of morphology and growth of transformed and normal fibroblasts. J. Natl. Canc. Inst. 48:1377-1387.

33. Teel, R. W. and Hall, R. G. (1973) Effect of dibutyryl cyclic AMP on the restoration of contact inhibition in tumor cells and its relationship to cell density and the cell cycle. Expt. Cell Res. 76:390-394.

34. Sheppard, J. R. (1971) Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3':5'-cyclic monophosphate. Proc. Natl. Acad. Sci. 68:1316-1320.

35. Dey, J., Vogel, A. and Pollack, R. (1974) Intracellular cyclic AMP concentration responds specifically to growth regulation by serum. Proc. Natl. Acad. Sci. 71:694-698.

36. Sheppard, J. R. and Prescott, D. M. (1972) Cyclic AMP levels in synchronized mammalian cells. Expt. Cell Res. 75:293-296.

37. Maganiello, V. and Vaughan, M. (1972) Prostaglandin E effects on adenosine 3':5'-cyclic monophosphate concentration and phosphodiesterase activities in fibroblasts. Proc. Natl. Acad. Sci. 69:269-273.

38. Strohl, W. A. (1969) The response of BHK21 cells to infection with type 12 adenovirus. II. Relationship of virus-stimulated DNA synthesis to other viral functions. Virology 39:653-665.

39. Johnson, G. S., Morgan, W. D. and Pastan, I. (1972) Regulation of cell motility by cyclic AMP. Nature 235:54-56.

40. Johnson, G. S. and Pastan, I. (1972) Cyclic AMP increases the adhesion of fibroblasts to substratum. Nature New Biol. 236:247-249.

41. Weber, M. J. (1973) Hexose transport in normal and in Rous Sarcoma virus-transformed cells. J. Biol. Chem. 248:2978-2983.

42. Venlita, S. and Rubin, H. (1973) Sugar transport in normal and Rous Sarcoma virus-transformed chick-embryo fibroblasts. Proc. Natl. Acad. Sci. 70:653-657.

43. Roller, B., Hirai, K. and Defendi, V. (1974) Effect of cAMP on nucleoside metabolism. I. Effect of thymidine transport and incorporation in monkey cells (CV-1). J. Cell PHys. 83:163-176.

44. Hauschka, P. V., Everhart, L. P. and Rubin, R. W. (1972) Alteration of nucleoside transport of Chinese hamster cells by dibutyryl cyclic monophosphate. Proc. Natl. Acad. Sci. 69:3542-3546.

45. Coggins, J. F., Johnson, G. S. and Pastan, I. (1972) The effect of dibutyryl cyclic adenosine monophosphate on synthesis of sulfated acid mucopolysaccharides by transformed fibroblasts. J. Biol. Chem. 247:5759-5764.

46. Roberts, R. M., Citorelli, J. J. and Walker, A. (1973) Fucose containing glycopeptides from the cell surface of Chinese hamster ovary cells grown in the presence or absence of cyclic AMP. Nat. New Biol. 244:86-89.

47. Roberts, R. M. and Baig, M. M. (1973) Comparative studies on the carbohydrate containing membrane components of normal and cAMP treated CHO-cells. Biochemical J. 134:329-339.

48. Schroder, C. H. and Hsie, A. W. (1973) Morphological transformation of enucleated Chinese hamster ovary cells by dibutyryl adenosine 3':5'-monophosphate. Nature New Biol. 246:58-60.

49. Carter, S. B. (1967) Effects of cytochalasins on mammalian cells. Nature 213:261-264.

50. Shay, J. W., Porter, K. R. and Prescott, D. M. (1974) The surface morphology and five structure of CHO-cells following enucleation. Proc. Natl. Acad. Sci. 71:3059-3063.

51. Patterson, D. and Waldren, C. A. (1973) The effect of inhibitors of RNA and protein synthesis on dibutyryl cyclic AMP mediated morphological transformations of CHO cells in order. Biochem. Biophys. Res. Commun. 50:566-573.

52. Rein, A., Carchman, R. A., Johnson, G. S. and Pastan, I. (1973) Simian virus 40 rapidly lowers cAMP levels in mouse cells. Biochem. Biophys. Res. Commun. 52:899-904.

53. Sheppard, G. R. (1973) Difference in cAMP levels in normal and transformed cells. Nature New Biol. 236:14-16.

54. Anderson, W. B., Lovelace, E. and Pastan, I. (1973) Adenylate cyclase activity is decreased in chick embryo fibroblasts transformed by wild type and temperature sensitive Schmidt-Ruppin Rous Sarcoma virus. Biochem. Biophys. Res. Commun. 52:1293-1299.

55. Anderson, W. B., Johnson, G. S. and Pastan, I. (1973) Transformation of chick-embryo fibroblasts by wild-type and temperature sensitive Rous Sarcoma virus alters adenylate cyclase activity. Proc. Natl. Acad. Sci. 70:1055-1059.

56. Burstin, S. J., Renger, H. C. and Basilico, C. (1974) Cyclic AMP levels in temperature sensitive SV40 transformed cell lines. J. Cell Phys. 84:69-74.

57. Zimmerman, J. E. and Raska, K. (1972) Inhibition of adenovirus type 12 induced DNA synthesis in G1 arrested BHK 21 cells by dibutyryl adenosine cyclic 3':5'-monophosphate. Nature New Biol. 239:145-147.

58. Criss, W. E. (1974) Second messenger system in neoplasia. Oncology 30:43-80.

59. Cuatrecasas, P. (1969) Interaction of insulin with the cell membrane: the primary action of insulin. Proc. Natl. Acad. Sci. 63:450-457.

60. Cuatrecasas, P. (1973) Insulin receptors of liver and fat cell membranes. Fed. Proc. 32:1838-1846.

61. Stoeckenius, W. and Engelman, D. M. (1969) Current models for the structure of biol membranes. J. Cell Biol. 42:613-646.

62. Winzler, R. J. (1969) Carbohydrates in cell surfaces. Int. Rev. of Cytol. 29:77-125.

63. Hendler, R. W. (1971) Biological membrane ultra structure. Physiological Rev. 51:66-96.

64. Wallach, D. F. H. (1972) The plasma membrane: dynamic perspectives genetics and pathology. The English Universities Press Ltd., London.

65. Steck, T. L. (1974) The organization of proteins in the human red blood cell membrane. J. Cell Biol. 62:1-19.

66. Singer, S. T. (1974) The molecular organization of membranes. Ann. Rev. of Biochem. 43:805-833.

67. Berlin, R. D., Oliver, J. N., Ukena, T. E. and Yin, H. H. (1975) The cell surface. New England J. of Med. 292:515-520.

68. Singer, S. J. and Nicolson, G. L. (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720-730.

69. Steck, T. L., Fairbanks, G. and Wallach, D. F. H. (1971) Disposition of major proteins in the isolated erythrocyte membrane: proteolytic dissection. Biochem. 10:2617-2624.

70. Wallach, D. F. H. (1972) The dispositions of proteins in the plasma membranes of animal cells: analytical approaches using controlled peptidolysis and protein labels. Biochim. Biophys. Acta 265:61-83.

71. Frye, L. D. and Edidin, M. (1970) The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. J. Cell Science 7:319-335.

72. Edidin, M. and Weiss, A. (1972) Antigen can formation in cultured fibroblasts: a reflection of membrane fluidity and cell motility. Proc. Natl. Acad. Sci. 69:2456-2459.

73. Edelman, G. M. (1973) Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. Proc. Natl. Acad. Sci. 70:1442-1446.

74. Albrecht-Buhler, G. and Solomon, F. (1974) Properties of particle movement in the plasma membrane of 3T3 mouse fibroblasts. Exp. Cell Res. 85:225-233.

75. Nicolson, G. L. and Yanadimachi, P. (1974) Mobility and restriction of mobility of plasma membrane lectin-binding components. Science 184:1294-1296.

76. Nicolson, G. L. (1971) Difference in topology of normal and tumor cell membranes shown by different surface distributions of ferretin conjugated Con A. Nature New Biol. 233:244-246.

77. de Petris, S. and Raff, M. C. (1973) Ligand-induced redistribution of concanavalin A receptors on normal, trypsinized and transformed fibroblasts. Nature New Biol. 244:275-278.

78. Rosenblith, J. L., Ukena, T. E., Yin, H. H., Berlin, R. D. and Kamovsky, M. J. (1973) A comparative evaluation of the distribution of concanavalin A-binding sites on the surfaces of normal, virally-transformed, and protease-treated fibroblasts. Proc. Natl. Acad. Sci. 70:1625-1629.

79. Inbar, M., Ben-Bassat, H., Fibach, E. and Sachs, L. (1973) Mobility of carbohydrate containing structures on the surface membrane and the normal differentiation of myeloid leukemic cells to macrophages and granulocytes. Proc. Natl. Acad. Sci. 70:2577-2581.

80. Kornberg, R. D. and McConnell, H. M. (1971) Inside-outside transitions of phospholipids in vesicle membranes. Biochem. 10:1111-1120.

81. Hiramo, H., Parkhouse, B., Nicolson, G. L., Lennox, E. S. and Singer, S. J. (1972) Distribution of saccharides residues on membrane fragments from myeloma cell homogenates. Proc. Natl. Acad. Sci. 69:2945-2949.

82. Steck, T. L. and Dawson, G. (1974) Topographical distribution of complex carbohydrates in the erythrocyte membrane. J. Biol. Chem. 249:2135-2142.

83. Byers, B. and Porter, K. R. (1964) Oriented microtubules in the elongation of cells of the developing lens rudiment after induction. Proc. Natl. Acad. Sci. 52:1091-1099.

84. Weber, K., Pollack, R. and Bibring, T. (1975) Antibody against tubulin: the specific visualization of cytoplasmic microtubules in tissue culture cells. Proc. Natl. Acad. Sci. 72:459-463.

85. Asthund, R. E. and Pastan, J. (1974) Myosin in cultured fibroblasts. J. Biol. Chem. 249:3903-3907.

86. Lazarides, E. and Weber, K. (1974) Actin Antibody: the specific visualization of actin filaments in non-muscle cells. Proc. Natl. Acad. Sci. 71:2268-2272.

87. Weber, K. and Groeschel-Stewart, U. (1974) Antibody to myosin: the specific visualization of myosin-containing filaments in nonmuscle cells. Proc. Natl. Acad. Sci. 71:4561-4564.

88. Piras, M. M. and Piras, R. (1974) Phosphorylation of vinblastin isolated microtubules from chick-embryonic muscles. Eur. J. Bioch. 47:443-452.

89. Allen, R. D. (1975) Evidence for linkages between microtubules and membrane bound vesicles. J. Cell. Biol. 64:497-503.

90. Ehrlich, H. P., Ross, R. and Bornstein, P. (1974) Effects of antimicrotubular agents on the secretion of collagen. A biochemical and morphological study. J. Cell. Biol. 62:390-405.

91. Berlin, R. D. and Uhena, T. E. (1972) Effect of colchicine and vinblastine on the agglutination of polymorphonuclear leucocytes by concanavalin A. Nature New Biol. 238:120-122.

92. Yin, H. H., Ukena, T. E. and Berlin, R. D. (1972) Effect of colchicine, colcemid and vinblastine on the agglutination by concanavalin A of transformed cells. Science 178:867-868.

93. de Petris, S. (1974) Inhibition and reversal of capina by cytoch. B. and vinblastine and colchicine. Nature 250:54-55.

94. Kyan, G. B., Borysenko, J. Z. and Karnovsky, M. J. (1974) Factors affecting the redistribution of surface-bound concanavalin A on human polymorphonuclear leukocytes. J. Cell. Biol. 62:251-365.

95. Nakamura, J. and Terayama, H. (1975) Colchicine affects kinetics of concanavalin A-mediated agglutination of hepatoma cells and plasma membranes from liver and hepatoma cells. Proc. Natl. Acad. Sci. 72:498-502.

96. Ukena, T. E., Borysenko, J. Z., Karnovsky, M. J. and Berlin, R. D. (1974) Effects of colchicine, cytochalasin B, and 2-deoxyglucose on the topographical organization of surface-bound concanavalin A in normal and transformed fibroblasts. J. Cell Biol. 61:70-82.

97. Oliver, J. M., Ukena, T. E. and Berlin, R. D. (1974) Effects of phagocytosis and colchicine on the distribution of lectin-binding sites on cell surfaces. Proc. Natl. Acad. Sci. 71:394-398.

98. Wickus, G., Gruenstein, E., Robbins, P. W. and Rich, A. (1975) Decrease in membrane-associated actin of fibroblasts after transformation by Rous Sarcoma Virus. Proc. Natl. Acad. Sci. 72:746-749.

99. Rambourg, A. (1971) Morphological and histochemical aspects of glycoproteins at the surface of animal cells. Int. Rev. of Cytol. 31:57.

100. Kraemer, P. M. (1971) Heparan sulfates of cultured cells. I. Membrane-associated and cell-sap species in Chinese hamster cells. Biochem. 10:1437-1445.

101. Nicolson, G. L. and Singer, S. J. (1974) The distribution and asymmetry of mammalian cell surface saccharides utilizing ferritin conjugated plant agglutinins as specific saccharide stains. J. Cell. Biol. 60:236-248.

102. Martinez-Palomo, A., Braislovsky, C. and Bernhard, W. (1969) Ultrastructural modifications at the cell surface and intracellular contacts of some transformed cell strains. Cancer Res. 29:925-937.

103. Torpier, G. and Montaguier, L. (1970) Ultrastructural changes of the surface of BHK 21/13 transformed cells, depending on adenine nucleotides. Int. J. Cancer 6:529-535.

104. Malucci, L., Poste, G. H. and Wells, V. (1972) Synthesis of cell coat in normal and transformed cells. Nature New Biol. 235:222-223.

105. Satoh, C., Duff, R., Rapp, F. and Davidson, E. A. (1973) Production of mucopolysaccharides by normal and transformed cells. Proc. Natl. Acad. Sci. 70:54-56.

106. Chiarugi, V. P., Vannucchi, S. and Urbano, P. (1974) Exposure of trypsin-removable sulphated polyanions on the surface of normal and cirally transformed BHK 21/C13 cells. Biochim. Biophys. Acta 345:283-293.

107. Hakomori, S. and Murakami, W. T. (1968) Glycolipids of hamster fibroblasts and derived malignant-transformed cell lines. Proc. Natl. Acad. Sci. 59:254-261.

108. Brady, R. O., Borek, C. and Bradley, R. M. (1969) Composition and synthesis of gangliosides in rat hepatocyte and hepatoma cell lines. J. Biol. Chem. 244:6552-6554.

109. Brady, R. O., Fishman, P. H. and Mora, P. T. (1973) Membrane components and enzymes in virally transformed cells. Fed. Proc. 32:102.

110. Brady, R. O. and Fishman, P. H. (1974) Biosynthesis of glucolipids in virus-transformed cells. Biochim. Biophys. Acta 355:121-148.

111. Hakomori, S. (1970) Cell density-dependent changes of glycolipid concentrations in fibroblasts, and loss of this response in virus transformed cells. Proc. Natl. Acad. Sci. 67:1741-1747.

112. Mora, P. T., Brady, R. O., Bradley, R. M. and McFarland, V. W. (1969) Gangliosides in DNA virus-transformed and spontaneously transformed tumorigenic mouse cell lines. Proc. Natl. Acad. Sci. 63:1290-1296.

113. Cumar, F. A. (1970) Enzymatic block in the synthesis of gangliosides in DNA virus-transformed tumorigenic mouse cell lines. Proc. Natl. Acad. Sci. 67:757-764.

114. Mora, P. T., Cumar, F. A. and Brady, R. O. (1971) A common biochemical change in SV40 and polyoma virus transformed mouse cells coupled to control of cell growth in culture. Virology 46:60-72.

115. Gahmberg, C. G. and Hakomori, S. (1974) Organization of glycolipids and glycoproteins in surface membranes: dependency on cell cycle and on transformation. Biochem. Biophys. Res. Commun. 59:283-291.

116. Gahmberg, C. G. and Hakomori, S. (1974) Surface carbohydrates of hamster fibroblasts. J. Biol. Chem. 250:2438-2446.

117. Gahmberg, C. G. and Hakomori, S. (1973) Altered growth behavior of malignant cells associated with changes in externally labeled glycoproteins and glycolipids. Proc. Natl. Acad. Sci. 70:3329-3333.

118. Wisnieksi, B. J., Williams, R. E. and Fox, C. F. (1973) Manipulation of F. A. composition in animal cells grown in culture. Proc. Natl. Acad. Sci. 70:3669-3673.

119. Sakiyama, H., Gross, S. K. and Robbins, P. W. (1972) Glycolipid synthesis in normal and virus-transformed hamster cell lines. Proc. Natl. Acad. Sci. 69:872-876.

120. Hynes, R. O. and Humphrys, K. C. (1974) Characterization of the external proteins of hamster fibroblasts. J. Cell Biol. 62:438-448.

121. Hynes, R. O. and Maepherson, J. (1974) In "Membrane Transformation in Neoplasia" eds. J. Schultz and R. E. Block, Miami White Symposium, Academic Press.

122. Grimes, W. J. (1970) Sialic acid transferases and sialic acid lvels in normal and transformed cells. Biochem. 9:5083-5092.

123. Den, H., Schultz, A. M., Basu, M. and Roseman, S. (1971) Glycosyltransferase activities in normal and polyoma-transformed BHK cells. J. Biol. Chem. 246:2721-2723.

124. Patt, L. M. and Grimes, W. J. (1974) Cell Surface glycolipid and glycoprotein glycosyltransferases of normal and transformed cells. J. Biol. Chem. 249:4159-4165.

125. Saito, M., Satoh, H. and Ukita, T. (1974) Sialyl transferase activities of rat ascites hepatoma cells and rat liver. Biochim. Biophys. Acta 362:549-557.

126. Roth, S. and White, D. (1972) Intercellular contact and cell-surface galactosyl transferase activity. Proc. Natl. Acad. Sci. 69:485-489.

127. Webb, G. C. and Roth, S. (1974) Cell contact dependence of surface galactosyltransferase activity as a function of the cell cycle. J. Cell Biol. 63:796-805.

128. Deppert, W., Werchau, H. and Walter, G. (1974) Differentiation between intracellular and cell surface glycosyl transferases: Gal transferase activity in intact cells and in cell homogenate. Proc. Natl. Acad. Sci. 71:3068-3072.

129. Truding, R., Shelanski, M. L., Daniels, M. P. and Morell, P. (1974) Comparison of surface membranes isolated from cultured murine neuroblastoma cells in the differentiated or undifferentiated state. J. Biol. Chem. 249:3973-3982.

130. Sharon, N. and Lis, H. (1972) Lectins: cell-agglutinating and sugar-specific proteins. Science 177:949-959.

131. Inbar, M. and Sachs, L. (1969) Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. Proc. Natl. Acad. Sci. 63:1418-1425.

132. Noonan, K. D. and Burger, M. M. (1973) The relationship of concanavalin A binding to lectin-initiated cell agglutination. J. Cell Biol. 59:134-142.

133. Noonan, K. D. and Burger, M. M. (1973) Binding of ^{3}H con A to normal and transformed cells. J. Biol. Chem. 248:4286-4292.

134. Bretscher, M. S. (1973) On labeling membranes. Nature New Biol. 245:114.

135. Rifkin, D. B., Compans, R. W. and Reich, E. (1972) A specific labeling procedure for proteins on the outer surface of membranes. J. Biol. Chem. 247:6432-6437.

136. Gahmberg, C. G. and Hakomori, S. (1973) External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J. Biol. Chem. 248:4311-4317.

137. Phillips, D. and Morrison, M. (1970) The arrangement of proteins in the human erythrocyte membrane. Biochem. Biophys. Res. Commun. 40:284-289.

138. Nagata, Y. and Burger, M. M. (1970) Wheat germ agglutinin isolation and chrystalization. J. Biol. Chem. 247:2248-2250.

139. Agrawal, B. I. and Goldstein, I. J. (1965) Specific binding of concanavalin A to cross linked dextran gels. Biochem. J. 96:23c-25c.

140. Leakly, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685.

141. Davis, B. J. (1964) Disc electrophoresis II method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121:404-427.

142. Turner, J. C. (1968) Triton X-100 scintillant for carbon-14 labeled materials. Int. J. of applied Rad. and Isot. 19:557-563.

143. Barland, P. and Schroeder, E. A. (1970) A new rapid method for the isolation of surface membranes from tissue culture cells. J. Cell Biol. 45:662-668.

144. Morrison, R. T. and Boyd, R. N., In "Organic Chemistry", 2nd edition, Section 6:6 and 28:18. Allyn and Bacon, Inc., Boston.

145. Brunette, D. M. and Till, J. E. (1971) A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. J. Membr. Biol. 5:215-224.

146. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275.

147. Ross, E. and Schatz, G. (1973) Assay of proteins in the presence of high concentrations of sulfhydryl compounds. Anal. Biochem. 54:304-306.

148. Gilman, A. G. (1970) A protein binding assay for adenosine 3':5'-cyclic monophosphate. Proc. Natl. Acad. Sci. 67:305-312.

149. Burger, M. M. and Goldberg, A. R. (1967) Identification of a tumor-specific determinant on neoplastic cell surfaces. Proc. Natl. Acad. Sci. 57:359-366.

150. Willingham, M. C. and Pastan, I. (1974) C-AMP mediates the Con A agglutinability of mouse fibroblasts. J. Cell Biol. 63:288-294.

APPENDIX

Program used for the calculation of percent of total DPM in samples labeled with ^{3}H and ^{14}C.

This program was written for a Wang 600 series calculator with a model 602 plotting output writer. This program will correct the ^{3}H counts for the overlap of ^{14}C counts into the ^{3}H channel, calculate and print DPM in each sample and will print and plot percent of total DPM for each sample.

Program:

STEP	CODE	KEY(S)	STEP	CODE	KEY(S)
0000	09 00	MARK	0026	03 06	-6
0001	00 00	EO	0027	00 15	CLEAR DISP
0002	00 14	CLEAR ALL	0028	09 03	STOP
0003	09 03	STOP	0029	06 06	ST 6
0004	06 00	ST 0	0030	07 00	RE 0
0005	09 03	STOP	0031	03 06	-6
0006	06 01	ST 1	0032	06 06	ST 6
0007	09 00	MARK	0033	05 07	÷7
0008	00 01	E1	0034	06 06	ST 6
0009	00 15	CLEAR DISP	0035	07 07	RE 7
0010	09 03	STOP	0036	03 07	-7
0011	06 05	ST 5	0037	00 15	CLEAR DISP
0012	07 00	RE 0	0038	09 03	STOP
0013	03 05	-5	0039	06 07	ST 7
0014	06 05	ST 5	0040	07 01	RE 1
0015	00 15	CLEAR DISP	0041	03 07	-7
0016	09 03	STOP	0042	06 07	ST 7
0017	06 06	ST 6	0043	00 15	CLEAR DISP
0018	07 00	RE 0	0044	09 03	STOP
0019	03 06	-6	0045	06 08	ST 8
0020	06 06	ST 6	0046	07 01	RE 1
0021	06 07	ST 7	0047	03 08	-8
0022	07 05	RE 5	0048	06 08	ST 8
0023	05 06	÷6	0049	07 07	RE 7
0024	06 05	ST 5	0050	05 08	÷8
0025	07 06	RE 6			

STEP	CODE	KEY(S)	STEP	CODE	KEY(S)
0051	06 07	ST 7	0101	07 09	RE 9
0052	00 15	CLEAR DISP	0102	03 08	-8
0053	09 03	STOP	0103	06 08	ST 8
0054	06 02	ST 2	0104	07 07	RE 7
0055	06 03	ST 3	0105	05 08	÷8
0056	00 01	E 1	0106	08 05	Jif+
0057	00 06	E 6	0107	00 15	CLEAR DISP
0058	06 04	ST 4	0108	00 00	EO
0059	09 00	MARK	0109	08 02	PRINT
0060	00 03	E 3	0110	10 02	F 2
0061	00 15	CLEAR DISP	0111	15 11	D11
0062	09 03	STOP	0112	06 04	ST 4
0063	06 08	ST 8	0113	06 15	ST 15
0064	07 00	RE 0	0114	06 14	ST 14
0065	03 08	08	0115	07 12	RE 12
0066	06 08	ST 8	0116	06 13	ST 13
0067	06 09	ST 9	0117	07 14	RE 14
0068	07 05	RE 5	0118	03 12	-12
0069	05 08	÷8	0119	08 05	Jif+
0070	08 05	Jif+	0120	07 14	RE 14
0071	00 15	CLEAR DISP	0121	06 13	ST 13
0072	00 00	EO	0122	07 13	RE 13
0073	08 02	PRINT	0123	06 12	ST 12
0074	05 02	÷2	0124	07 15	RE 15
0075	15 11	INDIR	0125	02 11	+11
0076	06 04	ST 4	0126	00 01	E1
0077	06 15	ST 15	0127	02 04	+4
0078	06 14	ST 14	0128	00 01	E1
0079	07 12	RE 12	0129	03 02	-2
0080	06 13	ST 13	0130	08 04	JifO
0081	07 14	RE 14	0131	08 00	SEARCH
0082	03 12	-12	0132	00 03	E3
0083	08 05	Jif+	0133	09 00	MARK
0084	07 14	RE 14	0134	00 04	E4
0085	06 13	ST 13	0135	07 10	RE 10
0086	07 13	RE 13	0136	08 02	PRINT
0087	06 12	ST 12	0137	06 02	ST 2
0088	07 15	RE 15	0138	06 10	ST 10
0089	02 10	+10	0139	00 01	E1
0090	00 01	E 1	0140	00 00	EO
0091	02 04	+ 4	0141	00 00	EO
0092	07 06	RE 6	0142	05 10	÷10
0093	05 09	÷9	0143	06 10	ST 10
0094	06 09	ST 9	0144	07 11	RE 11
0095	00 15	E 15	0145	08 02	PRINT
0096	09 03	STOP	0146	07 02	RE 2
0097	06 08	ST 8	0147	06 11	ST 11
0098	07 01	RE 1	0148	00 01	E 1
0099	03 08	-8	0149	00 00	E 0
0100	06 08	ST 8	0150	00 00	E 0

STEP	CODE	KEY(S)	STEP	CODE	KEY(S)
0151	05 11	÷11	0201	04 00	x0
0152	06 11	ST 11	0202	00 01	E1
0153	00 01	E1	0203	00 12	E12
0154	00 05	E6	0204	06 01	ST1
0155	06 04	St 4	0205	09 02	α
0156	09 00	MARK	0206	07 08	RE8
0157	00 05	E5	0207	02 02	+2
0158	07 11	RE 11	0208	07 00	RE0
0159	06 00	ST 0	0209	03 00	−0
0160	06 11	ST 11	0210	07 01	RE1
0161	07 00	RE 0	0211	03 01	−1
0162	05 12	÷12	0212	00 01	E1
0163	06 12	ST 12	0213	02 04	+4
0164	06 13	ST 13	0214	07 11	RE11
0165	00 04	E 4	0215	15 11	D11
0166	00 00	E0	0216	05 04	÷4
0167	00 00	E0	0217	06 06	ST6
0168	06 00	ST 0	0218	08 02	PRINT
0169	07 12	RE12	0219	10 02	+2
0170	05 00	÷0	0220	07 12	RE12
0171	06 12	ST12	0221	04 06	x6
0172	09 00	MARK	0222	07 06	RE6
0173	00 06	E6	0223	06 00	ST0
0174	00 00	E0	0224	00 00	E0
0175	06 00	ST0	0225	06 01	ST1
0176	00 00	E0	0226	09 02	α
0177	06 01	ST1	0227	05 09	÷9
0178	09 02	α	0228	02 02	+2
0179	05 06	÷0	0229	00 01	E1
0180	02 02	+2	0230	00 12	E12
0181	09 00	MARK	0231	04 00	x0
0182	00 07	E7	0232	00 01	E1
0183	07 10	RE10	0233	00 12	E12
0184	15 11	INDIR	0234	06 01	ST1
0185	05 04	÷4	0235	09 02	α
0186	06 05	ST5	0236	07 03	RE8
0187	08 02	PRINT	0237	02 02	+2
0188	05 02	÷2	0238	07 00	RE0
0189	07 12	RE12	0239	03 00	−0
0190	04 15	x5	0240	07 01	RE1
0191	07 15	RE5	0241	03 01	−1
0192	06 00	ST0	0242	07 05	RE5
0193	00 01	E1	0243	03 05	−5
0194	00 00	E0	0244	07 06	RE6
0195	06 01	ST1	0245	03 06	−6
0196	09 02	α	0246	00 01	E1
0197	05 06	÷6	0247	02 04	+4
0198	02 02	+2	0248	00 01	E1
0199	00 01	E1	0249	03 03	−3
0200	00 12	E12	0250	08 04	Jif0

STEP	CODE	KEY(S)
0251	08 00	SEARCH
0252	00 07	E7
0253	09 00	MARK
0254	00 08	E8
0255	00 00	E0
0256	06 00	STO
0257	00 09	E9
0258	06 01	ST1
0259	09 02	α
0260	05 06	÷6
0261	02 02	+2
0262	00 01	E1
0263	08 02	PRINT
0264	05 00	÷0
0265	00 00	E0
0266	08 02	PRINT
0267	10 00	FO
0268	00 14	CLEAR ALL
0269	09 14	END PROG

Once the program is in the memory of the calculator the following steps are required for the calculator to perform the calculations properly:

PRIME

SEARCH

0

^{14}C blank

go

^{3}H blank

go

^{14}C DPM IN STAND.

go

^{14}C CPM for STAND.

go

^{14}C overlap into ^{3}H channel

go

^{3}H DPM IN STAND.

go

^{3}H CPM

go

total number of samples

go

ENTER ^{14}C CPM for sample

go

ENTER ^{3}H CPM for same sample as ^{14}C entered above.

BIOGRAPHICAL SKETCH

Jacques van Veen was born on December 19, 1949, on Aruba, in the Netherlands Antilles, where he attended elementary and secondary schools. He was awarded a scholarship by the government of the Netherlands Antilles to study biochemistry and medicine in 1969, and enrolled at the University of South Carolina. In 1970 he transferred to the University of Florida, where in June, 1972, he received a Bachelor of Science degree in Chemistry. He received an award from the American Chemical Society's division of analytical chemistry. He entered graduate school in the Department of Biochemistry at the University of Florida in September, 1972. He will begin his studies in medicine at the Erasmus Universiteit in Rotterdam, the Netherlands, in September, 1975. The author is married to the former Brenda M. Every, who will receive the degree of Master of Arts in Economics from the University of Florida in August, 1975.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert M. Roberts, Chairman
Associate Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth D. Noonan
Assistant Professor Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Eugene G. Sander
Associate Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Bryan Gebhardt
Associate Professor of Pathology

This dissertation was submitted to the Graduate Faculty of the Department of Biochemistry in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1975

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Dean, Graduate School